

**BRADYKININ: VASOMOTOR TONE AND
ENDOGENOUS FIBRINOLYSIS IN MAN**

BY

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ABSTRACT

Bradykinin is a nonapeptide released into plasma during the contact phase of blood coagulation. It has a wide variety of physiological effects including vasodilatation, tissue-type plasminogen activator (t-PA) release, inflammatory mediator, ischaemic preconditioning and vasculogenesis. It is inactivated in plasma by angiotensin-converting enzyme (ACE). Inhibition of this enzyme has been shown to be beneficial in a variety of cardiovascular disorders including heart failure and hypertension, and it is clear that this benefit is not due entirely to reduction in the bioavailability of angiotensin II. We hypothesised that

- bradykinin is a vasodilator and stimulates endothelial t-PA release via a specific receptor and that this effect is augmented by ACE inhibition.
- in patients with heart failure, bradykinin contributes to peripheral and systemic vascular tone during treatment with ACE inhibition.

Forearm blood flow was measured using bilateral forearm plethysmography during intrabrachial drug infusion. Bilateral venous cannulae were inserted to perform blood sampling for estimation of plasma t-PA and plasminogen activator inhibitor 1 (PAI-1) concentrations. Cardiac output was measured with pulmonary artery catheterisation. The novel peptide bradykinin receptor antagonist, B9340, was used to oppose the effects of bradykinin.

Studies were performed in healthy volunteers

- to demonstrate the pharmacodynamics of B9340 and to demonstrate the selectivity of B9340 in opposing bradykinin-induced t-PA release.
- to demonstrate the safety and tolerability of systemic intravenous B9340 administration.

Studies were performed in patients with heart failure

- to demonstrate the effect of ACE inhibition on endothelial t-PA release.
- to demonstrate the effect of bradykinin antagonism on peripheral and systemic vascular tone in patients treated with ACE inhibition and angiotensin receptor blockade.

Results

In healthy volunteers

- Bradykinin and substance P caused dose-dependent vasodilatation in the infused forearm ($p < 0.001$). B9340 caused a dose-dependent inhibition of bradykinin-induced forearm vasodilatation and t-PA release ($p < 0.001$) without affecting substance P-induced vasodilatation or t-PA release ($p = \text{NS}$). B9340 caused a reversible inhibition of bradykinin-induced vasodilatation ($p < 0.001$) with a rapid onset and offset of action. Intravenous systemic B9340 administration inhibited the local bradykinin-induced forearm vasodilatation in a dose-dependent manner.

In patients with heart failure

- bradykinin and substance P caused dose-dependent vasodilatation and release of t-PA from the infused forearm ($p<0.05$). Long-term ACE inhibitor therapy caused an increase in forearm vasodilatation ($p<0.05$) and t-PA release ($p<0.001$) during bradykinin, but not substance P, infusion.
- incremental doses of B9340 caused a dose-dependent reduction in forearm blood flow ($p<0.01$). After withdrawal of ACE inhibitor therapy, B9340 produced no significant change in forearm blood flow.
- systemic intravenous B9340 administration resulted in greater mean arterial pressure, systemic vascular resistance, pulmonary arterial wedge pressure, and mean pulmonary arterial pressure during ACE inhibitor therapy compared with losartan therapy ($p<0.005$, $p<0.07$, $p<0.0001$, and $p<0.05$ respectively) or placebo infusion ($p<0.005$ for all).

We have shown that bradykinin is a potent vasodilator that stimulates endogenous t-PA release and that these effects are receptor specific and can be blocked by a bradykinin receptor antagonist. We have also shown that bradykinin does not contribute to peripheral or systemic vascular tone in health but does contribute to peripheral and systemic vascular tone in patients with heart failure treated with chronic ACE inhibition. We believe this suggests that many of the beneficial actions of ACE inhibition are mediated through bradykinin.

For Carol and Alexandra

CONTENTS

Abstract

Contents

Declaration

Acknowledgements

CHAPTER 1: INTRODUCTION

11 - 33

- 1.1 History of kallikrein and bradykinin
- 1.2 Bradykinin
- 1.3 The endogenous fibrinolytic system
- 1.4 Endothelium, function and dysfunction
- 1.5 Relationship between the renin-angiotensin and kinin systems
- 1.6 Hypotheses
- 1.7 Aims

CHAPTER 2: METHODOLOGY

34 - 50

- 2.1 Introduction
- 2.2 Forearm venous occlusion plethysmography
- 2.3 Measurement of systemic haemodynamics

2.4 General

2.5 Fibrinolytic and haemostatic parameters

CHAPTER 3: BRADYKININ RECEPTOR ANTAGONISM AND

51 - 64

ENDOTHELIAL TISSUE PLASMINOGEN ACTIVATOR RELEASE IN HUMANS

3.1 Summary

3.2 Introduction

3.3 Methods

3.4 Results

3.5 Discussion

3.6 Conclusions

CHAPTER 4: MARKED BRADYKIN-INDUCED TISSUE

65 - 80

PLASMINOGEN ACTIVATOR RELEASE IN PATIENTS WITH HEART

FAILURE MAINTAINED ON CHRONIC ACE INHIBITOR THERAPY

4.1 Summary

4.2 Introduction

4.3 Methods

4.4 Results

4.5 Discussion

4.6 Conclusions

**CHAPTER 5: BRADYKININ CONTRIBUTES TO THE
VASODILATOR EFFECTS OF CHRONIC ACE INHIBITION IN
PATIENTS WITH HEART FAILURE**

81 - 96

- 5.1 Summary
- 5.2 Introduction
- 5.3 Methods
- 5.4 Results
- 5.5 Discussion
- 5.5 Conclusion

**CHAPTER 6: BRADYKININ CONTRIBUTES TO THE SYSTEMIC
HAEMODYNAMIC EFFECTS OF CHRONIC ANGIOTENSIN-CONVERTING
ENZYME INHIBITION IN PATIENTS WITH HEART FAILURE**

97 - 114

- 6.1 Summary
- 6.2 Introduction
- 6.3 Methods
- 6.4 Results
- 6.5 Discussion
- 6.6 Conclusion

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS **115 - 130**

- 7.1 Bradykinin mediated stimulation of the endothelium
- 7.2 Angiotensin-converting enzyme inhibition and
tissue plasminogen activator
- 7.3 Angiotensin-converting enzyme inhibition and
plasminogen activator inhibitor
- 7.4 Bradkinin mediated vasodilatation
- 7.5 Haemodynamic effects of ACE inhibition and
angiotensin receptor blockade
- 7.6 Angiotensin receptor blockade and bradykinin
- 7.7 Future directions
- 7.8 Clinical relevance

REFERENCES **131 - 150**

APPENDIX **151 - 152**

Publications arising from or relevant to this thesis

DECLARATION

The work presented in this thesis was performed entirely by me except the systemic studies performed in Chapter 6 which were performed by Dr Nicholas Cruden, initially under my supervision, and the fibrinolytic assays in Chapters 3 and 4 which were performed by Pamela Dawson. Dr Ahmed Helmy performed eight forearm studies in Chapter 5. All figures are reproduced with permission.

Dr Fraser Witherow.

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CHAPTER 1

INTRODUCTION

1.1 HISTORY OF KALLIKREIN AND BRADYKININ

In 1925, in the course of research on the connection between cardiac and renal function, it was discovered that intravenous injection of dog urine into another dog decreased the blood pressure, as measured in the carotid artery with a Frank-Petter spring manometer. This hypotensive response could be reproduced using human urine and convinced the investigator, EK Frey, that he had discovered a previously unknown substance [Frey 1926]. As a surgeon, Frey was untrained in the field and enlisted the help of a chemist Heinrich Kraut to prepare a stable preparation [Kraut *et al* 1930]. This was achieved and the substance was known as 'F-stoff' after Frey, which later became kallikrein as it was found to be abundant in the pancreas. Kraut also found that the substance had an inactive precursor molecule later termed prekallikrein. It was later discovered that a plasma molecule of high molecular weight (HMW), when mixed with kallikrein produced an extremely potent vasodilator that caused profound hypotension in dogs. This was later termed kininogen.

In 1949 Rocha E Silva and co-workers were investigating the pharmacological effects of the venom of the snake *Borthops jararaca* (Figure 1.1) and discovered that incubation of the venom with the globulin fraction of plasma resulted in the formation of a potent vasodilator and smooth muscle stimulator [Rocha E Silva *et al* 1949]. The guinea-pig ileum they were using reacted slowly to the substance compared with histamine or acetylcholine and was thus named bradykinin. Its effect was also shown not to be inhibited by antihistamines or atropine proving it to be a new substance. The subsequent discovery of its structure and synthesis as a

nonapeptide took a further 12 years limited by the peptide chemistry of the day, but in 1960 Fox *et al* showed its effects as a potent vasodilator in man by delivering the drug via an intra-arterial infusion.



Figure 1.1 Borthops jararaca

1.2 BRADYKININ

1.2.1 STRUCTURE AND FUNCTION

The nonapeptide bradykinin (BK, Arg¹-Pro²-Pro³-Gly⁴-Phe⁵-Ser⁶-Pro⁷-Phe⁸-Arg⁹) is one of several endogenous oligopeptides called kinins. They are thought to act as local (paracrine) hormones, acting near the site at which they are generated. Bradykinin and the related decapeptide kallidin (Lys⁰-BK) are the major and the most important physiologically active components of the mammalian kinin family formed by the kallikrein-kinin system [Bhoola *et al* 1992; Blais *et al* 2000]. On the

basis of their function and regulation, two independent kallikrein-kinin systems can be distinguished in humans.

The actions of kinins are mediated by two G-protein-coupled, heptahelical receptor subtypes B₁ and B₂, distinguishable on the basis of tissue distribution and their pharmacological and molecular characteristics [Hess *et al* 1992; Menke *et al* 1994; Minshall *et al* 1995; Hall 1997]. The bradykinin B₂ receptors have high affinity for bradykinin and kallidin but low affinity for des-Arg⁹-bradykinin and are constitutively expressed, ready to respond to newly formed kinins [Regoli and Barabe 1980]. In contrast, bradykinin B₁ receptors, exhibit high affinity for des-Arg⁹-bradykinin and des-Arg⁹-kallidin but have a low affinity for bradykinin [Regoli *et al* 1998]. B₁ receptors are inducible, only being expressed in response to inflammation and tissue injury and are formed *de novo* in various cells including vascular endothelium [Raidoo *et al* 1997] by stimuli which activate the cytokinin system, particularly interleukin 1 β [Marceau 1995]. When activated by agonists B₂ receptors undergo desensitisation and internalisation. This contributes to the rapid reversibility of the biological effects *in vivo* [Austin *et al* 1997], whereas B₁ receptors are not internalised and not desensitised. (Table 1.1)

International Union of Pharmacology (IUPHAR) name	B1	B2
IUPHAR code	2.1:BK:1:B1:HUMAN:00	2.1:BK:2:B2:HUMAN:00
Alternate names	Bradykinin B1	Bradykinin B2
Amino acid composition	353	391
Gene/chromosome	<i>BDKRB1</i> /14q32.2	<i>BDKRB2</i> /14q32.2
Gene accession no	U12512	M88714
SwissProt accession no.	P46663	P30411
Selective agonist	Lys-des-Arg ⁹ -BK	BK
Selective antagonist	Lys-[Leu ⁸]des-Arg ⁹ -BK	Icatibant
Primary G protein coupling	G α_q /G α_i	G α_q /G α_i
Primary regulation		
Expression	Induction	Constitutive
Signaling	Limited desensitization	Extensive desensitization

Table 1.1 Human Bradykinin Receptor Nomenclature

The majority of the prominent physiological actions of bradykinin seem to be mediated by the stimulation of the bradykinin B₂ receptor [Hall 1997], and include activation of sensory nerve terminals, induction of the release of pro-inflammatory and hyperalgesic mediators such as neuropeptides, leukotrienes and cytokines, increased vascular permeability and induction of vasodilatation [Calixto *et al* 2000]. These pharmacological effects are the underlying cause of the strong pro-inflammatory and nociceptive properties of bradykinin, and it is believed that excessively increased local bradykinin concentrations contribute significantly to diseases like septic shock, pancreatitis, oedema, asthma, rhinitis, colitis, arthritis and pain [Hall 1997; Heitsch 1999; Bock and Longmore 2000; Heitsch 2000; Heitsch 2002]. In contrast to these long known ‘classical’ pro-inflammatory properties of kinins, they have also been identified to significantly contribute to powerful organoprotective including cardioprotective mechanisms. It has been shown that

bradykinin via B₂ receptors causes the release of vasoactive molecules such as nitric oxide [Cockcroft *et al* 1994a; O’Kane *et al* 1994], prostaglandins (PGI₂ and PGE₂), endothelium-derived hyperpolarising factor [Nakashima *et al* 1993; Miura *et al* 1999] and tissue-type plasminogen activator (t-PA) [Labinjoh *et al* 2001]. It also triggers ischaemic preconditioning [Lessar *et al* 1999] and the translocation of the glucose transporter GLUT4 [Rett *et al* 1996].

1.2.2 SYNTHESIS AND RELEASE

The plasma kinin forming system consists of two essential plasma proteins, prekallikrein and HMW kininogen, that interact once bound to an endothelial cell surface receptor complex that contains cytokeratin 1, urokinase plasminogen activator (u-PA) receptor and gC1qR. Previously it was thought that when factor XII is activated to XIIa, it converted plasma prekallikrein to kallikrein and kallikrein digested HMW kininogen to liberate the nonapeptide bradykinin. However recent work shows the interaction of HMW kininogen with its endothelial cell surface receptor is key to the regulated activation of prekallikrein, which circulates in plasma complexed to HMW kininogen. Work with cultured endothelial cells suggests HMW kininogen bound prekallikrein is rapidly converted to kallikrein by the enzyme prolylcarboxypeptidase in a process that is independent of factor XII [Motta *et al* 1998; Rojkaer *et al* 1998]. On cultured endothelial cells and cell matrices, factor XII activation occurs subsequent to prekallikrein activation (Rojkaer *et al* 1998; Motta *et al* 2001). Activation of prekallikrein, unlike that of factor XI, can therefore proceed in the absence of factor XIIa [Shariat-Madar *et al* 2001] (Figure 1.2).

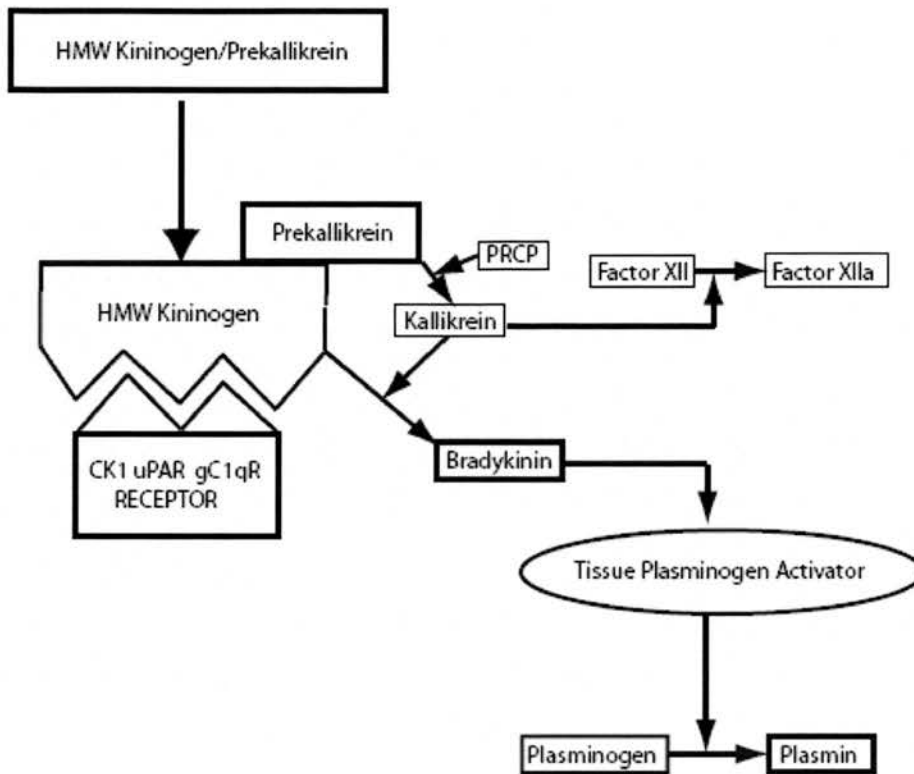


Figure 1.2 Assembly and activation of the plasma kallikrein-kininogen system on endothelial cells. Plasma prekallikrein (PK) circulates in complex with high molecular weight kininogen (HK). The HK•PK complex binds to a multiprotein receptor complex that consists of cytokeratin 1 (CK1), urokinase plasminogen activator receptor (uPAR) and gC1qR. The proteins of the HK•PK receptor complex co-localise on endothelial cell membranes. When HK•PK binds to endothelial cells, PK is rapidly converted to kallikrein (K) by the enzyme prolylcarboxypeptidase (PRCP), which is constitutively active on endothelial cell membranes. The resulting kallikrein autodigests its receptor, HK, to liberate bradykinin (BK), which can liberate tissue plasminogen activator (tPA) from endothelial cells. Kallikrein also activates factor XII (FXII), which binds to the same multiprotein receptor complex as HK in its absence.

Factor XII is synthesised in the liver and circulates as a single-chain zymogen with no detectable enzymatic activity [Silverberg and Diehl 1987]. It is a large protein with 596 amino acids and contains domains homologous to fibronectin, plasminogen and plasminogen activator [Cool *et al* 1985; Castellino and Beals 1987].

In contrast to factor XII, prekallikrein does not circulate as a separate protein but is bound to HMW kininogen in a 1:1 bimolecular complex [Bock *et al* 1985]. It is this complex that binds to its endothelial receptor surfaces initiating 'contact activation' primarily through HMW kininogen. Some of the complex dissociates during contact binding allowing bradykinin formation to occur both locally and at sites distant from the initiating reaction [Cochrane and Revak 1980]. High molecular weight kininogen acts as a coagulation cofactor that can bind factor XI and prekallikrein, and factor XII may be activated when bound to the same receptor as the HMW kininogen receptor.

Tissue Pathway

Bradykinin may also be generated via a tissue pathway where a prokallikrein is converted to tissue kallikrein by as yet uncharacterised enzymes and tissue kallikrein is secreted locally to digest low molecular weight (LMW) kininogen to generate lysyl-bradykinin (kallidin), and a plasma aminopeptidase converts kallidin to bradykinin [Guimaraes *et al* 1973]. Tissue prekallikrein can be converted to tissue kallikrein in plasma by both plasmin and plasma kallikrein [Takada *et al* 1985; Takahashi *et al* 1986]. Tissue kallikrein is immunologically and structurally unrelated to plasma kallikrein and is secreted by various organs or cells such as salivary glands, kidney, pancreas, prostate, pituitary gland and neutrophils [Ole-MoiYoi *et al* 1979; Powers and Nasjletti 1982; Figueroa *et al* 1989]. Its primary substrate is LMW kininogen but can cleave HMW kininogen to release kallidin, which is functionally similar to bradykinin but of lower potency.

1.2.3 CLEARANCE

Regulation of contact activation occurs via plasma protease inhibitors mainly C1 inhibitor, which is a major inhibitor of factor XIIa, factor XIa and kallikrein but is not active against other coagulation enzymes [de Agostini et al 1984]. Kallikrein is also inhibited by α 2-macroglobulin [McConnell 1972]. In plasma, bradykinin is first digested by carboxypeptidase N, which removes the C-terminal arginine to leave des-Arg⁹-bradykinin [Sheikh and Kaplan 1989] and then by angiotensin-converting enzyme (ACE) (kininase II) via its tripeptidase activity (its dipeptidase activity converts angiotensin I to angiotensin II) [Sheikh and Kaplan 1986] to a pentapeptide and a tripeptide that are both thought to be inactive. As the pulmonary circulation contains a large amount of ACE 90-95% of bradykinin is inactivated as it passes through the lungs [Bonner *et al* 1990].

1.2.4 FLETCHER TRAIT

Bradykinin's intimate relationship with plasminogen activator can be seen in patients with Fletcher trait who are deficient in prekallikrein and therefore cannot form bradykinin via the plasma pathway. This rare disorder is associated with an increase in myocardial infarction and cerebrovascular accident [Currimbhoy *et al* 1976; Harris *et al* 1989; Hess *et al* 1991; Kabasawa *et al* 1991].

1.3 THE ENDOGENOUS FIBRINOLYTIC SYSTEM

Activation of the fibrinolytic system results in the conversion of the inactive proenzyme, plasminogen to plasmin, the active enzyme. Plasmin degrades fibrin into

soluble fibrin degradation products thus maintaining the patency of blood vessels. In addition to specific degradation of fibrin, plasmin is probably also involved in the cleavage of a variety of proteins on cell surfaces and in the extracellular matrix. All the enzymes of the fibrinolytic system are serine proteases (their catalytic site contains the amino acids serine, aspartate, histidine), while the inhibitors of fibrinolysis are members of the serine protease inhibitors ('serpins') superfamily [Lijnen and Collen 1995].

1.3.1 PLASMINOGEN

Plasminogen is a 92 kD single-chain glycoprotein, which on activation is converted to plasmin, a two-chain protein with proteolytic activity. The amino-terminal A-chain has five kringle domains which mediate the binding of plasminogen to fibrin (where it is subsequently activated) and the interaction with $\alpha 2$ -antiplasmin. The B-chain contains the proteolytic active site. The main physiologic activators of plasminogen are t-PA and u-PA. The main synthesis site for u-PA is the kidney. u-PA is released as a 54 kD single-chain molecule (scu-PA), which has little catalytic activity and which is cleaved to produce the two-chain molecule (tcu-PA) with a 100-fold increased plasminogen activating capacity [Lijnen *et al* 1990]. Urokinase plasminogen activator mediated activation seems to be mainly involved in pericellular proteolysis whereas plasminogen activation via t-PA is mainly involved in the dissolution of fibrin in the blood vessels.

1.3.2 TISSUE PLASMINOGEN ACTIVATOR

Tissue plasminogen activator is synthesised by and released from endothelial cells into the circulating blood as a single-chain molecule with a molecular mass of 68 kD [van Hinsbergh 1998]. The activation of plasminogen by t-PA occurs on the fibrin surface and on the endothelial cell surface. This process allows for efficient and localised plasminogen activation, since the catalytic activity of surface-bound t-PA is much higher than in solution [Hoylaerts *et al* 1982; Plow *et al* 1991].

The inhibitors of the fibrinolytic systems are α 2-antiplasmin, plasminogen activator inhibitor 1 (PAI-1) and plasminogen activator inhibitor 2 (PAI-2). α 2-antiplasmin is a 70 kD single-chain glycoprotein which reacts with plasmin by formation of a 1:1-molar reversible complex, followed by covalent binding which results in an irreversible complex. The surface-bound plasmin molecules are protected from the action of α 2-antiplasmin and are inactivated 100-1000 times slower than free plasmin molecules.

In the absence of fibrin, t-PA has very weak activity as a plasminogen activator. However, once bound to fibrin, the catalytic activity of t-PA rises a 1000-fold due to conformational changes resulting from the binding of the finger and second kringle domains [van Zonneveld *et al* 1986], and the formation of a ternary complex between plasminogen, fibrin and t-PA [R nby 1982].

Release of tPA occurs in response to ischaemia in the vasculature and fibrin formation along with direct stimulation by mediators such as bradykinin and substance-P.

Genetic factors can influence the ability of the endothelium to release tPA in response to direct stimulation. Patients with the ACE DD genotype have been shown to have impaired tPA release in response to bradykinin stimulation in the coronary microvasculature, [Ohira *et al* 2004] and black Americans have impaired vascular vasomotor responses to bradykinin compared to white Americans although their tPA release is similar suggesting different receptor sensitivity to bradykinin between these two groups. [Rosenbaum *et al.* 2002]

Disease states can also influence endothelial tPA release. Patients with chronic renal disease have impaired tPA release in response to DDAVP stimulation despite preserved vasomotor function. [Hrafnkelsdóttir *et al* 2004] and an increase in tPA expression was seen in cultured endothelial cells incubated with simvastatin suggesting that cholesterol lowering with statins may improve tPA release. [Bourcier and Libby 2000]

1.3.3 PLASMINOGEN ACTIVATOR INHIBITOR

Plasminogen activator inhibitor 1 is a 52 kD single-chain glycoprotein which is synthesised in a variety of cells such as endothelial cells, smooth muscle cells, platelets and hepatocytes. It is stabilised in plasma by binding to vitronectin [Declerck *et al* 1988b] which preserves its active conformation and protects it against oxidation [Lindahl *et al* 1988]. The plasminogen activator inhibitor 1 concentration

in plasma exhibits a diurnal variation with a peak in the morning and a trough in the evening. This variation is opposed to the circadian variation of the t-PA plasma levels.

Plasminogen activator inhibitor 1 is a rapid inhibitor of t-PA and of t-PA but does not inhibit scu-PA. The reaction pattern is similar to that of plasmin inhibition by α_2 -antiplasmin and t-PA bound to fibrin is protected from rapid inhibition by PAI-1 just as surface-bound plasmin is protected from inhibition by α_2 -antiplasmin. Plasminogen activator inhibitor 2 is produced in the placenta and in leukocytes, and is released into the blood stream during pregnancy. It primarily inhibits t-PA and its physiological role remains incompletely understood [Kruithof *et al* 1995]. However, recent reports suggest that PAI-2 may play a significant role in cell proliferation and differentiation [Yu *et al* 2002], and in the cellular defence mechanisms against viral infections [Shafren *et al* 1999]. Plasminogen activator inhibitor 2 also modulates the expression of adhesion molecules and may inhibit cell motility and metastasis [Mueller *et al* 1995].

The clearance of t-PA predominantly occurs through the removal of both active t-PA and t-PA/PAI-1 complexes from the circulation by the liver [Chandler *et al* 1997]. This clearance process is rapid, with plasma half-lives for active t-PA and t-PA/PAI-1 complexes of 2.4 and 5.0 minutes respectively. In the presence of high PAI-1 activity, more t-PA is complexed and the total clearance is slowed due to the longer half-life of the t-PA/PAI-1 complex. The pharmacodynamics of t-PA clearance suggest a two compartment model [Chandler *et al* 1997] and it is likely that

endothelial cell t-PA receptors contribute significantly to this effect [Cesarman *et al* 1994].

1.4 ENDOTHELIUM, FUNCTION AND DYSFUNCTION

In 1980, Furchgott and Zawadzki reported that the endothelial cells play an obligatory role in the relaxations elicited by acetylcholine in isolated arteries of the rabbit. This pivotal observation has since been extended to a wide variety of arteries from different species, including humans [Furchgott and Vanhoutte 1989; Lüscher and Vanhoutte 1990]. Furchgott, [Furchgott 1988] and Ignarro and colleagues [Ignarro *et al* 1987; Ignarro *et al* 1988] proposed that the endothelial mediator involved in endothelium-dependent relaxation was nitric oxide which stimulates soluble guanylate cyclase in vascular smooth muscle cells leading to increased levels of cyclic guanosine monophosphate (cyclic GMP), and thus relaxation. Moncada and co-workers [Palmer *et al* 1987, Palmer *et al* 1988, Palmer and Moncada 1989] demonstrated that indeed nitric oxide was the major endothelium-derived relaxing factor. However, nitric oxide is not the sole mediator of endothelium-dependent relaxations. In certain blood vessels, prostacyclin, by activating the production of cyclic adenosine monophosphate (cyclic AMP), can contribute, as can another, as yet undescribed, endothelium-derived hyperpolarising factor. Since these seminal works it has been widely recognised that the vascular endothelium acts as a regulator of vascular function by synthesising and releasing a vast array of mediators that affect not only vasomotion, but cellular proliferation, haemostasis and inflammation in response to a variety of stimuli.

1.4.1 REGULATION OF VASCULAR TONE

The endothelium plays a central role in the regulation of vascular tone by balancing synthesis and release of the vasodilators nitric oxide, prostacyclin and endothelium-derived hyperpolarising factor with synthesis and release of vasoconstrictors such as endothelin. This balance of vasoactive mediators is altered in response to various stimuli such as bradykinin, acetylcholine and angiotensin II. Both nitric oxide and endothelin are continuously released by the endothelium to regulate basal vascular tone [Vallance *et al* 1989; Haynes and Webb 1994], and their release is affected by both endocrine and paracrine sources of vasoactive mediators. Dysfunction of the endothelium results in impaired regulation of vascular tone, endogenous fibrinolysis and inflammation leading to hypertension and atherosclerosis [Ross 1999].

1.4.2 REGULATION OF FIBRINOLYSIS AND HAEMOSTASIS

The regulation of fibrinolysis has been discussed previously in this chapter. The endothelium provides receptors for several substances involved in endogenous fibrinolysis. In addition, it synthesises von Willebrand factor, which is a cofactor that stabilises factor VIII:C and facilitates platelet adhesion to the subendothelial matrix.

1.4.3 ENDOTHELIAL DYSFUNCTION AND ATHEROSCLEROSIS

Dysfunction of the endothelium in the forearm vascular bed has been shown to be present in cardiovascular diseases such as hypertension [Linder *et al* 1990; Panza *et al* 1990] and diabetes [Makimattila *et al* 1999], as well as patients with risk factors for atheromatous vascular diseases such as hypercholesterolaemia [Creager *et al*

1990; Chowienczyk *et al* 1992], and smoking [Heitzer *et al* 1996 Newby *et al* 1999]. It has been proposed that endothelial dysfunction occurs as a generalised phenomenon in response to injury of the vasculature and the subsequent complex inflammatory response to injury leads to inflammatory cell adhesion to the endothelium with subsequent migration into the vessel wall [Ross 1999].

Strong evidence of a role for endothelial dysfunction as a marker of atherosclerotic risk stems from several studies investigating the association between the presence of endothelial dysfunction in both the coronary and systemic circulations and prognosis. A recent multivariate analysis of current studies describing the association between coronary or peripheral endothelial dysfunction and cardiovascular events demonstrated that endothelial dysfunction is strongly and independently associated with cardiovascular events. This analysis included nearly 2,500 patients, with duration of follow-up between 1 and 92 months. The nature of the cardiovascular events varied among the studies but overall represented major cardiovascular events such as cardiac death, myocardial infarction, and need for revascularisation. Of note in this analysis was the similar power of coronary and peripheral endothelial dysfunction to predict cardiovascular events, and that cardiovascular events may occur remotely from the site in which endothelial dysfunction was detected, underscoring the systemic nature of endothelial dysfunction [Lerman and Zeiher 2005].

1.5 RELATIONSHIP BETWEEN THE RENIN-ANGIOTENSIN AND KININ SYSTEMS

The renin-angiotensin-aldosterone system plays a pivotal role in controlling vascular tone and blood pressure but it also has an intimate relationship with the kininogen-kinin system. As can be seen from Figure 1.3 the main link between the two systems is ACE. This enzyme converts angiotensin I to angiotensin II activating one system and cleaves bradykinin to its breakdown products inactivating the opposing system.

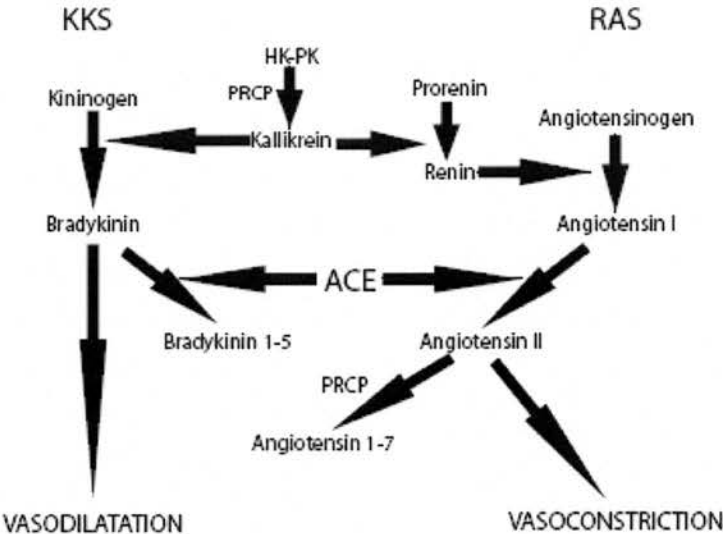


Figure 1.3 Interaction of the plasma kallikrein/kinin system (KKS) with the renin-angiotensin system (RAS). HK, high molecular weight kininogen; PK, prekallikrein; PRCP, prolylcarboxypeptidase; HKa, plasma kallikrein-cleaved, high molecular weight kininogen free of bradykinin; ACE, ANG I converting enzyme; tPA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor 1.

Recently, a homologue of ACE, angiotensin-converting enzyme 2 (ACE 2), has been recognised [Bernstein 2002; Crackower *et al* 2002]. Angiotensin-converting

enzyme 2 has different substrate specificity than ACE. Angiotensin-converting enzyme 2 is a carboxypeptidase mainly located in the heart, kidney, and testis. It degrades angiotensin I by removing the COOH-terminal lysine, making the peptide angiotensin (1-9), which has been reported to enhance arachidonic acid release by bradykinin and resensitise the B₂ receptor [Marcic *et al* 1999; Bernstein 2002]. Alternatively, ACE degrades angiotensin I by proteolysing it at the penultimate phenylalanine to produce angiotensin II (angiotensin 1-8). Angiotensin-converting enzyme 2 is kinetically 100-fold greater than prolylcarboxypeptidase at degrading angiotensin II to angiotensin (1-7) [Ody *et al* 1978; Vickers *et al* 2002]. Thus, in those tissues where ACE 2 is present, it is the preferred angiotensinase. The two converting enzymes also have different specificities to bradykinin. ACE 2 does not degrade bradykinin, but degrades des-Arg⁹-bradykinin at its carboxy terminal amino acid [Donoghue *et al* 2000].

Further links between the two systems can be seen in their opposing effects on the endogenous fibrinolytic system. In cultured endothelial cells, angiotensin II upregulates and stimulates the release of PAI-1 [Vaughan *et al* 1995], although direct infusion of angiotensin II into the human forearm does not produce any acute change in either t-PA or PAI-1 antigen release or activity [Labinjoh *et al* 2001]. Bradykinin infusion on the other hand produces a marked release of t-PA from the endothelium and has been shown to be one of the most potent stimulants of t-PA release [Labinjoh *et al* 2001].

In addition, further commonalities between the systems can be seen in the fact that plasma kallikrein may be able to activate prorenin [Derkx *et al* 1987] and that both systems have effects on angiogenesis [Emanueli *et al* 2002; Colman *et al* 2003].

1.5.1 RENIN-ANGIOTENSIN SYSTEM MANIPULATION AND CLINICAL EFFECTS

The outstanding benefits of ACE inhibitor therapy can be seen in a variety of clinical settings. After showing that it resulted in blood pressure lowering, ACE inhibitor therapy was tested in patients with heart failure and resulted in improved survival and improved symptoms [The CONSENSUS Trial Study Group 1987]. Since that time patients at lower and lower risk from atheromatous vascular disease have been shown to benefit from ACE inhibition (Table 1.2).

Study Name	Drug	Condition	Primary Endpoint
SAVE	Captopril	Heart Failure	Mortality
SOLVD	Enalapril	Heart Failure	Mortality
CONSENSUS	Enalapril	Heart Failure	Mortality
V-HeFT	Enalapril	Heart Failure	Mortality
ISIS -4	Captopril	Post MI	Mortality
AIRE	Ramipril	Post MI	Mortality
TRACE	Trandolapril	Post MI	Mortality
GISSI - III	Lisinopril	Post MI	Mortality
HOPE	Ramipril	Vasculopathy	CV death, MI, CVA
EUROPA	Perindopril	Vasculopathy	CV death, MI, cardiac arrest
PEACE	Trandolapril	Vasculopathy	CV death, MI, revascularisation

Table 1.2 Some of the trials of ACE inhibition in cardiovascular disease. MI – myocardial infarction, CV – cardiovascular, CVA – cerebrovascular accident.

Although the clinical benefits of ACE inhibitor therapy were initially thought to be due to blockade of angiotensin II production, it became clear that ACE inhibition had effects beyond the renin-angiotensin system. It was shown that plasma levels of angiotensin II returned to pre-treatment levels after chronic ACE inhibitor therapy in patients with hypertension, suggesting that there were non ACE dependent methods of angiotensin II generation [Mento and Wilkes 1995]. This led to the hope that drugs directed at blocking the direct action of angiotensin II on its receptor would produce greater clinical benefits. Unfortunately in most clinical conditions where ACE inhibitor therapy has been compared directly with an angiotensin receptor blocker there has been a trend towards greater morbidity and mortality benefit with ACE inhibition [ELITE II 2000; OPTIMAAL 2002], although when very high doses of angiotensin receptor blocking agents were used in patients with heart failure, valsartan was found to be non inferior to captopril [Pfeffer MA *et al* 2003]. These results would suggest that the additional benefit of ACE inhibitor therapy may be due to non angiotensin effects and a major part of this may be due to manipulation of the kinin system.

1.6 HYPOTHESES

A model to assess endothelium-dependent vasomotion and endogenous t-PA release, has been published extensively [Newby *et al* 1997] and will be used to test the following hypotheses.

1. The vasodilator actions of bradykinin in healthy volunteers are dependent on the bradykinin B₂ receptor and can be blocked by a novel bradykinin receptor antagonist B9340.
2. The stimulation of t-PA release from the endothelium by bradykinin in healthy volunteers is dependent on the bradykinin B₂ receptor and can be blocked by a novel bradykinin receptor antagonist B9340.
3. Endogenous t-PA release is augmented in patients with heart failure treated with chronic ACE inhibitor therapy.
4. Bradykinin does not contribute to basal vascular tone in healthy volunteers or patients with heart failure.
5. Bradykinin contributes to basal vascular tone in patients with heart failure treated with chronic ACE inhibitor therapy.

1.7 AIMS

The aims of the thesis are:

CHAPTER 3.

In healthy volunteers

- to establish the pharmacodynamics, and tolerability of intra-arterial bradykinin, an endothelium-dependent vasodilator, in the forearm.
- to determine the tolerability and pharmacodynamics characteristics of B9340, a novel bradykinin receptor antagonist.

- to determine the contribution of the endothelial bradykinin receptor to stimulated t-PA release *in vivo* in humans.
- to establish the selectivity of B9340, by comparing the vasomotor and fibrinolytic responses of intra-arterial bradykinin with those of substance P.

CHAPTER 4.

In patients with heart failure

- to determine the effect of intra-arterial infusion of bradykinin, substance P, and sodium nitroprusside on vasomotion and t-PA release.
- to determine if endogenous t-PA release is augmented by chronic ACE inhibitor therapy.

CHAPTER 5.

In healthy volunteers

- to confirm that B9340 is a potent and specific bradykinin receptor antagonist *in vivo*.
- to establish the role of bradykinin in the maintenance of peripheral vascular tone.

In patients with heart failure

- to determine whether endogenous bradykinin contributes to the maintenance of basal peripheral vascular tone using B9340 and the selective bradykinin B₂ receptor antagonist HOE-140.

CHAPTER 6.

In healthy volunteers

- to evaluate the safety and tolerability of intravenous doses of B9340 in healthy male volunteers using an ascending dose design.
- to demonstrate that the bradykinin receptor antagonist, B9340, inhibits bradykinin activity in the systemic circulation.

In patients with heart failure

- to determine whether endogenous bradykinin contributes to the haemodynamic effects of long-term ACE inhibition and angiotensin II receptor blockade.

CHAPTER 2

METHODOLOGY

2.1 INTRODUCTION

When examining *in vivo* vascular responses to pharmacological agents in man, drug administration can be systemic or local. Systemic delivery causes concomitant effects on organs, such as the brain, kidney and heart, and influences neurohumoral reflexes through changes in systemic haemodynamics. Because of these confounding influences, vascular responses cannot be wholly attributed to a direct effect of the drug on the vascular bed [Webb 1995]. Unilateral brachial artery infusions combined with bilateral venous occlusion plethysmography provides a method of directly assessing peripheral vascular responses without invoking these systemic influences. However measuring systemic vascular responses, with a pulmonary artery catheter, has the benefit of assessing the total effect of the drug on all systemic vascular beds rather than just a single site and is helpful in confirming systemic vascular responses are similar to those response evoked locally. Both techniques are employed in the following studies.

2.2 FOREARM VENOUS OCCLUSION PLETHYSMOGRAPHY

The use of venous occlusion plethysmography to measure blood flow in humans was first described over 90 years ago by Hewlett and van Zwaluwenburg [1909]. The technique is relatively simple with the venous return from the forearm being briefly interrupted by inflating a cuff, placed around the upper arm, to well above venous pressure but below diastolic pressure. An inflation pressure of 40 millimetres of mercury (mmHg) is used for intervals of 10 seconds, followed by 5 seconds of

deflation, which does not alter arterial inflow and allows venous emptying [Wilkins and Bradley 1946]. The forearm is positioned above the level of the heart to ensure adequate venous emptying during the period of deflation. This is achieved by resting the elbows on foam pads and supporting the hands with pillows. The hands are excluded from the circulation during measurements by the rapid inflation of smaller cuffs, placed around the wrists, to well above systolic pressure (220 mmHg for normotensive subjects). The wrist cuffs are inflated for 60 seconds before starting measurements of flow in order to allow forearm blood flow (FBF) to stabilise [Kerslake 1949]. The hands are excluded from the system as they contain a large number of arteriovenous shunts and are very sensitive to temperature variations.

Changes in forearm volume are measured with a plethysmograph, which consists of mercury in silastic strain gauges [Ensink and Hellige 1981]. The strain gauges are placed around the widest part of the forearm, and act as resistors connected as one arm of a Wheatstone bridge [Whitney 1953]. Alterations in forearm blood flow represent alterations in forearm resistance vessels in the muscle and skin. Changes in forearm volume result in a corresponding change in arm circumference and strain gauge length, which can be detected as an alteration in electrical resistance of the gauge, and thus potential difference [Ensink and Hellige 1981]. The gauge length is made equal to the resting circumference of the limb, so that changes in limb volume are directly proportional to the changes in resistance [Hokanson *et al* 2000]. This provides a measure of blood flow to that part of the forearm enclosed by the two cuffs. This is expressed as millilitres per 100 millilitres of forearm volume per

minute, when electronic calibration is employed [Whitney 1953; Hokanson *et al* 2000].

Changes in FBF can be reported either in absolute terms or as a percentage of baseline values. In the following studies, the ratio of FBF in the infused arm to the control arm (usually around 1) is quoted, as a percentage change from baseline. It has the theoretical advantage of minimising the influences of small changes in FBF affecting both arms due, for example, to alteration in sympathetic activation or blood pressure [Benjamin *et al* 1995]; the non-infused arm acting as a contemporaneous control. Percentage changes in the infused FBF are calculated [Benjamin *et al* 1995; Webb 1995] as follows:

$$\% \text{ Change in blood flow} = 100 \times (I_t/NI_t - I_b/NI_b) / I_b/NI_b$$

where I_b and NI_b are the infused and non-infused FBF at baseline (time 0) respectively, and I_t and NI_t are the infused and non-infused FBF at a given time point respectively.

2.3 MEASUREMENT OF SYSTEMIC HAEMODYNAMICS

Since the advent of the pulmonary flotation catheter [Swan *et al* 1970], invasive measurement of cardiac output (CO) and thus systemic and pulmonary vascular resistance has changed from a research tool to routine clinical practice in critical care

areas. The invasive methods of measuring CO are more reliable than non-invasive measurements using thoracic bioimpedance [Leslie *et al* 2004].

2.3.1 CARDIAC OUTPUT

Cardiac output is defined as the amount of blood ejected per unit of time, usually expressed in litres per minute (L/min). The range of 'normal' CO is difficult to define because it is influenced by several variables, most importantly body size. Because CO seems to be predominantly a function of the body's oxygen consumption or metabolic rate which is thought to correlate best with body surface area (BSA), it has become customary to express CO in terms of cardiac index ((L/min)/body surface area, m²)).

$$\text{BSA (m}^2\text{)} = 0.007184 \times \text{weight}^{0.425} \text{ (kg)} \times \text{height}^{0.725} \text{ (cm)}$$

By using cardiac index rather than CO it greatly reduces variation in range of normal values. Cardiac output is also affected by age, as metabolic rate reduces in old age, posture as CO decreases approximately 10% when rising from lying to sitting and approximately 20% from lying to standing. Cardiac output can also be affected by temperature, anxiety, heat, and humidity amongst other factors.

There are currently two main methods of measuring CO, the Fick oxygen technique, sometimes called the 'direct' Fick method, and the indicator dilution method. Both techniques resemble each other and are based on the theoretical principle described by Adolph Fick in 1870 [Fick 1870]. The principle is that the amount of a substance

taken up or released by an organ is the product of its blood flow rate and the difference in the concentration of the substance between the organ's arterial and venous blood.

Fick Oxygen or 'Direct' Fick Method

In the Fick oxygen method [Fick 1870], pulmonary blood flow is determined by measuring the arteriovenous difference of oxygen across the lungs and the rate of oxygen uptake by blood from the lungs. Assuming no intracardiac shunting, the pulmonary blood flow is equal to systemic blood flow, thus $CO = \text{oxygen consumption} / \text{arteriovenous oxygen difference}$.

Arteriovenous Oxygen Difference

Measurement of the arteriovenous oxygen difference can be performed by sampling blood from the pulmonary artery and from the pulmonary veins (although more commonly left ventricular or systemic arterial blood is used as it is easier to sample), and measuring the oxygen saturation of the samples. The theoretical oxygen carrying capacity (TC) is required and can be calculated from Haemoglobin (g/L) x C where C is a constant normally given the value 1.34 or 1.36, thus

$$\text{A-V oxygen difference} = (\% \text{saturation}^{\text{SA}} \times \text{TC}) - (\% \text{saturation}^{\text{PA}} \times \text{TC})$$

where SA and PA are systemic artery and pulmonary artery respectively.

Oxygen Consumption

Measurement of oxygen consumption requires measuring the fractional contents of oxygen in room air and patients' expired air and volume and rate of inspiration and expiration, however in actual practice it is often assumed that oxygen consumption can be predicted from the body surface area thus oxygen consumption is 125 mL/m^2 or 100 mL/m^2 for older patients.

Indicator dilution method

The indicator dilution method is a specific application of Fick's general principle and was introduced by Stewart in 1897 [Stewart 1897]. There are two types of indicator dilution method, continuous infusion and single bolus methods with single bolus being most widely used.

Fundamental requirements for the indicator dilution method are

- A bolus injection of non-toxic indicator that mixes completely with blood and whose concentration can be measured accurately.
- The indicator is neither added to nor subtracted from between injection and sampling sites.
- Most of the indicator must pass the site of sampling before recirculation begins.
- The indicator substance must go through a portion of the central circulation where all the blood of the body becomes mixed.

Thermodilution method

In the thermodilution method the non-toxic indicator (I) is cold dextrose solution, the injection site is the right atrium. The temperature of the indicator (T_i) is measured at injection and the temperature of blood (T_b) is measured in the pulmonary artery as a function of time (t). Using the following equation cardiac output by thermodilution (CO_{td}) in mL is given as

$$CO = \frac{V_i(T_b - T_i)(S_i \cdot C_i / S_b \cdot C_b)60}{\int \Delta T_b(t)}$$

where V_i is the volume of the injectate (mL), and S_b , S_i , C_b and C_i are the specific gravity and specific heat of blood and injectate respectively. When 5% dextrose is used as an indicator, $(S_i \cdot C_i / S_b \cdot C_b) = 1.08$.

Potential Errors

Some of the injectate may warm in the catheter after the temperature has been measured this may give a mild overestimation of CO. In low flow, low output states warming of the indicator may occur between right atrium and pulmonary artery also giving a slight overestimation of the CO. The method is also unreliable in the presence of significant tricuspid regurgitation.

2.3.2 MEASUREMENT OF VASCULAR RESISTANCE

The applicability of laws such as Poiseuille's law derived from steady state fluid mechanics in assessing vascular resistance is somewhat ambiguous because blood flow is pulsatile, blood is a nonhomogeneous fluid and the vascular bed is a non-

linear, elastic, frequency dependent system. In such a system resistance varies continuously with pressure and flow and is influenced by many factors, such as inertia, reflected waves, and the phase angle between pulse and flow wave velocities [O'Rourke 1982; Milnor 1989].

Despite these hurdles, systemic and pulmonary vascular resistance are routinely calculated in clinical and research settings, from invasive haemodynamic measurements. The formula generally used for systemic vascular resistance is

$$\text{Systemic Vascular Resistance} = (\text{Mean arterial pressure} - \text{Mean right atrial pressure}) / \text{systemic blood flow}.$$

The systemic blood flow is volume flow i.e. cardiac output, expressed in L/min, and pressures are expressed in mmHg. These equations yield resistance in arbitrary resistance units expressed in mmHg per L/min, also called Wood units. They can be converted to metric units expressed as dynes-sec-cm⁻⁵ by multiplying the pressure difference by 80.

The systemic vascular resistance can also be adjusted for body size to give the systemic vascular resistance index by substituting the cardiac index for the CO in the previous equation.

The thermodilution method used in the following studies involved a catheter with a thermistor element proximally and a temperature sensor distally, giving a continuous

measure of CO [Ditmyer *et al* 1995; Bottiger *et al* 1996]. This avoids the need for repeated injections of cold dextrose thus, reducing intravenous volume loading during the study and eliminating intra-operator variability in cold injection technique. Although termed 'continuous', absolute measurements are taken every 30 seconds using the 'STAT' mode as this correlates better with the bolus thermodilution method, especially during acute haemodynamic changes [Lazor MA *et al* 1997].

2.4 GENERAL

2.4.1 ETHICAL CONSIDERATIONS

All studies were undertaken in accordance with the Declaration of Helsinki of the World Medical Association and with the approval of the Local Research Ethics Committee. The written informed consent of each subject or patient was obtained before entry into the study.

2.4.2 SUBJECT PREPARATION

None of the normal healthy volunteers and controls received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study. All subjects and patients abstained from alcohol for 24 hours and from food, tobacco and caffeine-containing drinks for at least 5 hours before each study. Studies were performed in a quiet, temperature controlled room maintained at 21.5-23.5°C.

2.4.3 BLOOD PRESSURE MEASUREMENT

During forearm venous occlusion plethysmography, blood pressure was monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) [Wiinberg *et al* 1988].

2.4.4 BRACHIAL ARTERY CANNULATION

The brachial artery of the non-dominant arm was cannulated with a 27-gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% lidocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia. The cannula was attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK) and patency maintained by infusion of saline (0.9%: Baxter Healthcare Ltd, Thetford, UK) via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 mL/min (Figure 2.1).



Figure 2.1 Brachial Artery Cannulation, with strain gauge in position around the forearm.

2.4.5 BLOOD FLOW MEASUREMENT

Blood flow was measured in the infused and non-infused forearms by venous occlusion plethysmography as described above. Analogue voltage output from an EC-4 strain gauge plethysmograph (D.E. Hokanson) was processed by a MacLab[®] analogue-to-digital converter and Chart[™] v3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto an iMAC computer (Apple Computers Inc, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph. (Figure 2.2).

2.4.6 DATA ANALYSIS

Plethysmographic data were extracted from the Chart™ data files and FBF were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 97; Microsoft). Usually, the last five flow recordings in each 3 minute measurement period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point: in effect using the non-infused arm as a contemporaneous control for the infused arm [Benjamin *et al* 1995; Webb 1995].

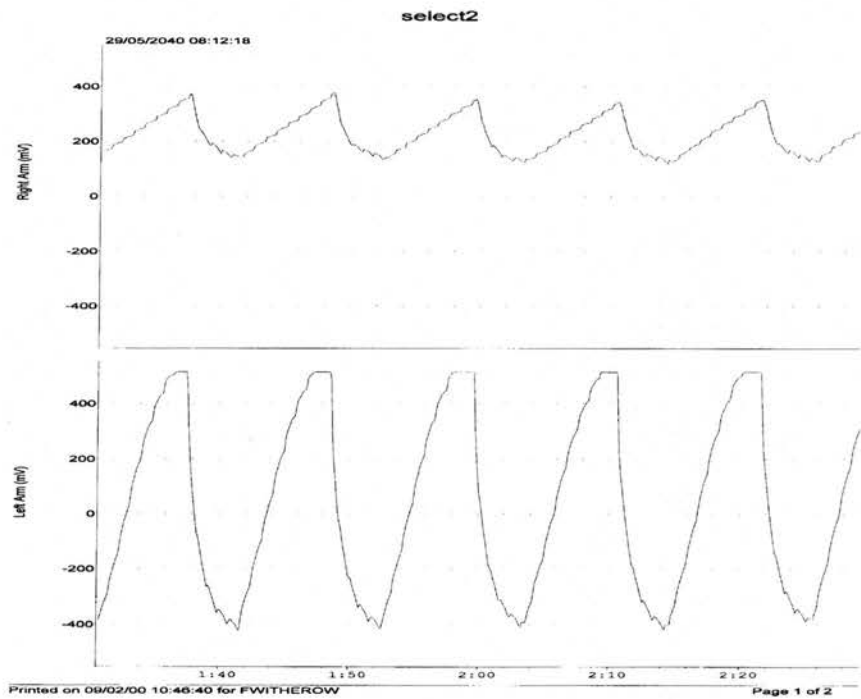


Figure 2.2 Typical plethysmographic data. The upslope of the waveform represents the rate of forearm swelling. The right arm is the control arm and the left arm is the infused arm during bradykinin infusion.

2.4.7 PULMONARY ARTERY CATHETER INSERTION

With aseptic technique, a 8-French venous sheath (Vygon GmbH&Co.KG, Prager Ring, Aachen, Germany) was inserted via the right femoral vein under local anaesthesia (Xylocaine; Astra Pharmaceuticals Ltd). Under fluoroscopic screening, cardiac rhythm monitoring and pressure monitoring, a continuous CO thermodilution Swan-Ganz catheter (Edwards Lifesciences, Irvine, Calif) was positioned with its distal tip in the pulmonary arterial tree. To allow for the systemic and pulmonary haemodynamics to return to a steady basal level after catheter insertion, a 50 minute infusion of saline at 1 mL/min was given prior to commencing the study. Pulmonary arterial pressure, pulmonary arterial wedge pressure, and central venous pressure were recorded at each time point using a Hewlett Packard monitor (U78339A; Hewlett Packard, Andover, Mass). Continuous cardiac output was recorded using a Vigilance monitor (Edwards Lifesciences, Irvine, Calif). Heart rate and blood pressure were measured non-invasively using a semi-automated sphygmomanometer (U78339A; Hewlett Packard, Andover, Mass).

2.5 FIBRINOLYTIC AND HAEMOSTATIC PARAMETERS

2.5.1 VENOUS SAMPLING

In the forearm studies venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossa in both arms as described previously [Plumpton *et al* 1995]. Ten millilitres of blood were withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden) and citrate (Monovette®, Sarstedt, Nümbrecht, Germany) tubes, and kept on

ice before being centrifuged at 2,000 *g* for 30 minutes at 4°C. Platelet free plasma was decanted and stored at -80°C before assay [Kluft and Verheijen 1990].

In the systemic study venous blood was sampled from the femoral vein sheath. Blood samples were collected on ice, into lithium heparin (Monovette®) for plasma ACE activity and ethylene diamine tetraacetic acid tubes (Monovette®) for angiotensin II analysis and centrifuged immediately, the resulting supernatant stored at -70°C until assayed.

2.5.2 PLASMA FIBRINOLYTIC PARAMETER ASSAYS

Plasma PAI-1 and t-PA antigen concentrations were determined using an enzyme-linked immunosorbent assay; Coaliza® PAI-1 [Declerck *et al* 1988a] and Coaliza® t-PA [Booth *et al* 1987] (Chromogenix AB, Mölndal, Sweden) respectively. Plasma PAI-1 and t-PA activities were determined by a photometric method, Coatest® PAI-1 [Wiman *et al* 1988] and Coaset® t-PA [Gram *et al* 1987] (Chromogenix AB). Intra-assay coefficients of variation were 7.0% and 5.5% for t-PA and PAI-1 antigen, and 4.0% and 2.4% for activity, respectively. Inter-assay coefficients of variability were 4.0%, 7.3%, 4.0% and 7.6% respectively. The sensitivities of the assays were 0.5 ng/mL, 2.5 ng/mL, 0.10 IU/mL and 5 AU /mL respectively.

2.5.3 PLASMA NEUROHORMONE ANALYSIS

Plasma ACE activity was determined using colorimetric spectrophotometry (SIGMA Diagnostics, St Louis, Mo) [Holmquist *et al* 1979]. After extraction using Bond Elut columns (Varian; Harbor City, Calif), plasma angiotensin II (Diasorin, Stillwater,

Minn) concentrations were determined by radioimmunoassay [Morton and Webb 1985].

2.5.4 HAEMATOCRIT MEASUREMENT

Haematocrit was determined by capillary tube centrifugation of blood anticoagulated by ethylene diamine tetraacetic acid.

2.5.5 DATA ANALYSIS AND STATISTICS

Estimated net release of t-PA activity and antigen was defined as the product of the infused FBF (based on the haematocrit, Hct and the infused FBF and the concentration difference between the infused ($[t\text{-PA}]_{\text{Inf}}$) and non-infused arms ($[t\text{-PA}]_{\text{Non-inf}}$).

$$\text{Estimated net forearm t-PA release} = \text{FBF} \times (1 - \text{Hct}) \times (t\text{-PA}_{\text{Inf}} - t\text{-PA}_{\text{Non-inf}})$$

Data were examined by analysis of variance (ANOVA) with repeated measures, 2-tailed paired Student's *t*-test and Student's unpaired *t*-test using Excel 97 (Microsoft). All results are expressed as mean \pm standard error of the mean. Statistical significance was taken at the 5% level.

Power calculations were not performed for the forearm studies because when power calculations are applied to studies with large physiological changes in blood flow, the sample sizes required are meaningless (normally only one or two subjects are required if the power calculations are followed.)

A power calculation was performed for the patient study in chapter six. For a power of 80% to detect a 3.5 mmHg change in MAP a sample size of 6 was required. We studied more patients to increase the reliability of the data.

CHAPTER 3

BRADYKININ RECEPTOR ANTAGONISM AND ENDOTHELIAL TISSUE PLASMINOTEN ACTIVATOR RELEASE IN HUMANS



3.1 SUMMARY

Objective - We sought to assess pharmacodynamic responses to the bradykinin antagonist B9340 and to determine the contribution of the endothelial bradykinin receptor to stimulated t-PA release in humans.

Methods and Results - Bilateral forearm blood flow and plasma fibrinolytic variables were measured in eight volunteers during 100 minutes of intrabrachial infusions of saline placebo, B9340 at 4.5 nmol/min, or B9340 at 13.5 nmol/min. On each occasion, intra-arterial bradykinin (30-3000 pmol/min) and substance P (4-6 pmol/min) were coinfused for 10 minutes at each dose. To assess the onset and offset of action, six additional subjects on two occasions received intra-arterial bradykinin (100 pmol/min) for 60 minutes with a coinfusion of either saline placebo or B9340 (13.5 nmol/min) for 12 minutes. During placebo infusion, bradykinin and substance P caused dose-dependent vasodilatation in the infused forearm ($p < 0.001$). B9340 caused a dose-dependent inhibition of bradykinin-induced forearm vasodilatation and t-PA release ($p < 0.001$) without affecting substance P-induced vasodilatation or t-PA release ($p < \text{NS}$). B9340 caused a reversible inhibition of bradykinin-induced vasodilatation ($p < 0.001$) with a rapid onset and offset of action.

Conclusions - B9340 is a potent, reversible, and selective competitive receptor antagonist of bradykinin-induced vasodilatation and t-PA release in humans.

3.2 INTRODUCTION

Bradykinin is an endogenous, vasoactive, nonapeptide mediator involved in many physiologic processes. It is cleaved from HMW kininogen during the contact phase of blood coagulation [Reddigari and Kaplan 1988], resulting in endothelium-dependent vasodilatation and stimulation of t-PA release from human endothelial cells [van den Eijnden-Schrauwen *et al* 1995]. It has a brief duration of action (plasma half-life of 15-30 seconds) owing to its rapid degradation by several enzymes, principally ACE. Bradykinin appears to contribute to the vascular effects of ACE inhibitor therapy in hypertension [Gainer *et al* 1998] and heart failure [Witherow *et al* 2001].

Bradykinin receptor antagonists have been developed from peptide analogues of bradykinin. The most widely used bradykinin receptor antagonist is HOE-140, or icatibant, which demonstrates high selectivity for the B₂ receptor. Recently, a third generation synthetic peptide antagonist of bradykinin, B9340, has been synthesised. It has a similar chemical structure to HOE-140 and differs by replacement of the α -(2-indanyl)glycine at position 7 of the molecule with a tetrahydroisoquiniline-3-carboxylic acid moiety. In comparison with HOE-140, B9340 retains similar potency of inhibition at the bradykinin B₂ receptor (median inhibitory concentration [IC₅₀] of 0.158 nmol/L for both) but has greater inhibition at the B₁ receptor (IC₅₀ of 1000 nmol/L and 7.9 nmol/L for HOE-140 and B9340, respectively [Stewart *et al* 1997]).

The purpose of this study was to assess pharmacodynamic responses to the novel bradykinin antagonist B9340 and to determine the contribution of the endothelial bradykinin receptor to stimulated t-PA release *in vivo* in humans. To assess the selectivity of B9340, we compared the vasomotor and fibrinolytic responses of bradykinin with those of substance P, a bradykinin receptor independent, endothelium-dependent vasodilator and stimulator of t-PA release [Newby *et al* 1999].

3.3 METHODS

3.3.1 STUDY SUBJECTS

Fourteen healthy, male volunteers were recruited into the study, which was performed with approval of the Local Research Ethics Committee, in accordance with the Declaration of Helsinki (1989) and the written, informed consent of each subject. Each volunteer was studied at the same time of day and had been fasting for at least 4 hours before each study. All subjects avoided alcohol for 24 hours and caffeine containing products for 5 hours before study. No medications or vasoactive drugs were taken in the 7 days before each study.

3.3.2 INTRA-ARTERIAL ADMINISTRATION

All studies were performed in a quiet, temperature controlled (22-25°C) room. On each occasion, the brachial artery of the non-dominant arm was cannulated with a 27-gauge needle (Cooper's Needle Works Ltd) after 1% lidocaine local anaesthesia. The needle was attached to a 16-gauge epidural catheter (Portex Ltd), and needle patency

was maintained by an infusion of 0.9% saline at 1 mL/min. The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 mL/min.

3.3.3 FOREARM BLOOD FLOW AND BLOOD PRESSURE

Forearm blood flow was measured by mercury in silicone elastomer strain gauge venous occlusion plethysmography, as previously described [Webb 1995; Petrie *et al* 2000]. Immediately after each FBF measurement, pulse and blood pressure were measured non-invasively in the non-infused arm throughout each study with a semi-automated digital sphygmomanometer (UA-731, A&D Engineering). Mean arterial pressure was defined as the diastolic blood pressure plus one-third of the pulse pressure.

3.3.4 DRUGS

Pharmaceutical grade B9340 (Clinalfa AG), substance P (Clinalfa), and bradykinin (Clinalfa) were dissolved in 0.9% saline before infusion. All solutions were freshly prepared on the day of study. The doses of bradykinin were based on previous studies [Labinjoh *et al* 2000] and the doses of B9340 based on the dose of HOE-140 given in other studies [Cockroft *et al* 1994a] as it has a similar efficacy at the B2 receptor.

3.3.5 VENOUS SAMPLING AND ASSAYS

Venous cannulas (17-gauge) were inserted into large subcutaneous veins of the antecubital fossa in both arms, as described previously [Newby *et al* 1997]. Ten millilitres of blood were withdrawn simultaneously from each arm and collected into

acidified, buffered citrate (Biopool Stabilyte) and citrate (Monovette, Sarstedt) tubes and kept on ice before being centrifuged at 2,000 g for 30 minutes at 4°C. Platelet free plasma was decanted and stored at -80°C before assay. Plasma t-PA antigen and activity concentrations were determined by ELISA (Coaliza t-PA, Chromogenix AB) and a photometric method (Coatest t-PA, Chromogenix AB), respectively, as described previously [Newby *et al* 1998].

3.3.6 STUDY DESIGN

Protocol 1: Efficacy and Selectivity of B9340

Eight healthy, male volunteers attended on three occasions at least 1 week apart. After 30 minutes equilibration with saline infusion, intra-arterial placebo (0.9% saline), B9340 at 4.5 nmol/min, or B9340 at 13.5 nmol/min was infused for 100 minutes on separate occasions in a randomised, double-blind, ascending dose design. Placebo/B9340 was coinfused with saline for 10 minutes; bradykinin at 30, 300, and 3,000 pmol/min for 10 minutes at each dose; and after a 30 minute saline infusion, substance P at 4, 8, and 16 pmol/min for 10 minutes at each dose. Forearm blood flow and mean arterial pressure were measured every 10 minutes for the duration of the study. Venous samples were obtained at baseline, after 10 minutes of B9340/placebo infusion, and after each dose of bradykinin and substance P.

Protocol 2: Onset and Offset of B9340 Action

Six additional healthy, male volunteers attended on two occasions at least 1 week apart. After 30 minutes equilibration with saline infusion, intrabrachial bradykinin was infused at 100 pmol/min for 60 minutes. After 12 minutes of bradykinin infusion,

B9340 (Clinalfa) at 13.5 nmol/min or placebo (saline vehicle) was coinjected for 12 minutes in a randomised, double-blind manner. Forearm blood flow and mean arterial pressure were measured every 6 minutes for the duration of the study.

3.3.7 DATA ANALYSIS AND STATISTICS

Plethysmographic data were extracted from the software (Chart) data files, and FBF was calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 97, Microsoft). The last five flow recordings in each 3 minute measurement period were calculated and averaged for each arm. The effective dose, causing a 100% increase in FBF (ED_{100}), was calculated to assess the degree of bradykinin antagonism. The percentage increase in blood flow was determined from the ratio of the FBF's, as described previously [Petrie *et al* 2000].

Estimated net release of t-PA antigen and activity was defined as the product of the infused FBF (based on the haematocrit [Hct] and the infused FBF), and the concentration difference between the infused ($t\text{-PA}_{\text{Inf}}$) and non-infused forearms ($t\text{-PA}_{\text{Non-inf}}$).

$$\text{Estimated net t-PA release} = \text{FBF} \times (1\text{-Hct}) \times (t\text{-PA}_{\text{Inf}} - t\text{-PA}_{\text{Non-inf}})$$

Data were examined, where appropriate, by ANOVA with repeated measures and 2-tailed, paired Student's *t*-test with commercially available software (Excel 97). All results are expressed as mean \pm SEM. Statistical significance was taken at the 5% level.

3.4 RESULTS

The infusions were well tolerated with no major adverse events. There were no significant changes in FBF in the non-infused arm, heart rate, or blood pressure during the infusions.

3.4.1 PROTOCOL 1: EFFICACY AND SELECTIVITY OF B9340

Subjects were aged 25 ± 5 years with a body mass index of 22 ± 2 kg/m².

Forearm Blood Flow

Baseline blood flow during saline infusion was 2.0 ± 0.2 mL \cdot 100 mL⁻¹ \cdot min⁻¹ in the infused arm and 2.0 ± 0.1 mL \cdot 100 mL⁻¹ \cdot min⁻¹ in the non-infused arm. In comparison with placebo, B9340 infusion alone caused no significant change in the infused FBF from baseline at either dose (placebo, $-7.4 \pm 3.7\%$; B9340 at 4.5 nmol/min, $8.1 \pm 7.6\%$; and B9340 at 13.5 nmol/L, $3.8 \pm 5.5\%$; ANOVA, ($p=0.8$). During placebo infusion, bradykinin and substance P caused dose-dependent vasodilatation in the infused forearm (ANOVA, $p<0.001$ for both; Figure 3.1). At doses of 4.5 and 13.5 nmol/min, B9340 caused 7-fold and 18-fold increases in the ED₁₀₀ for bradykinin, respectively (ANOVA, $p<0.001$ for both; Figure 3.1). In contrast, B9340 had no effect on substance P-induced vasodilatation (Figure 3.1).

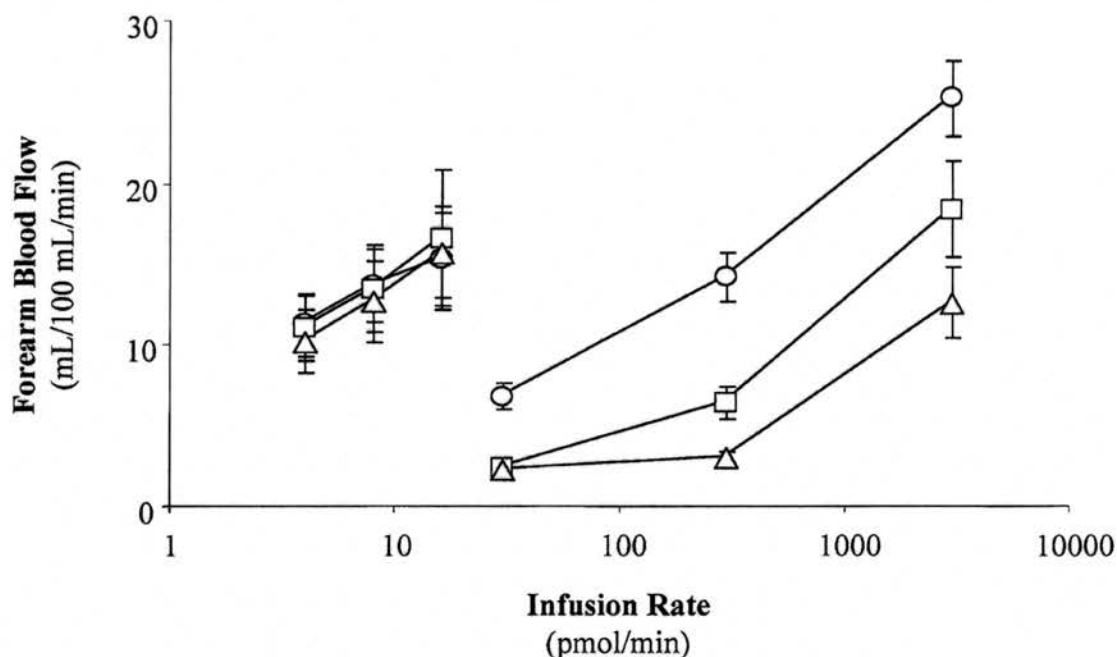


Figure 3.1 Infused FBF during substance P (left) and bradykinin (right) infusion with saline placebo (circles), B9340 at 4.5 nmol/min (squares), and B9340 at 13.5 nmol/min (triangles). $P < 0.001$, ANOVA, for all blood flow responses; $p < 0.001$, ANOVA, for comparisons between saline placebo and B9340 administration for bradykinin infusion (right) only.

Estimated Net t-PA Release

Baseline plasma t-PA antigen and activity concentrations during saline infusion were 3.4 ± 0.4 ng/mL and 1.3 ± 0.3 IU/mL in the infused arm and 3.6 ± 0.3 ng/mL and 1.3 ± 0.3 IU/mL in the non-infused arm, respectively. B9340 infusion alone produced no change in plasma t-PA antigen or activity (ANOVA, $p = 0.9$). Both bradykinin and substance P produced a dose-dependent increase in plasma t-PA antigen and activity concentrations in the infused forearm (ANOVA, $p < 0.001$). Both doses of B9340 completely inhibited t-PA antigen and activity release at 300 pmol/min bradykinin and reduced t-PA antigen and activity release at 3,000 pmol/min by 4- to 8-fold (ANOVA, $p < 0.001$ for both doses; Figure 3.2). There was no effect of B9340 on substance P- induced t-PA release (Figure 3.2).

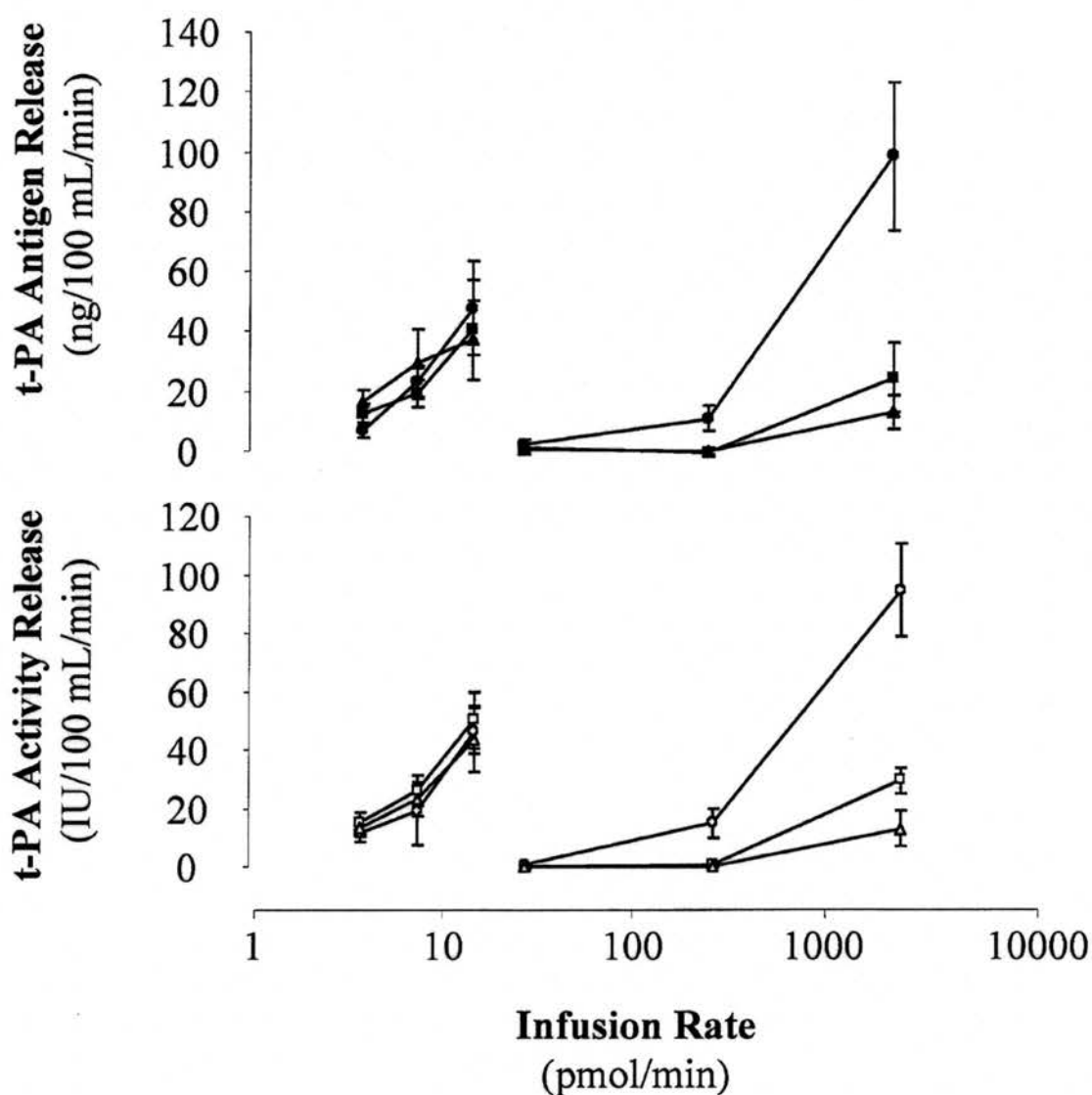


Figure 3.2 Estimated net t-PA antigen (closed symbols, top) and activity (open symbols, bottom) release during substance P (left) and bradykinin (right) infusion with saline placebo (circles), B9340 at 4.5 nmol/min (squares), and B9340 at 13.5 nmol/min (triangles). $P < 0.05$, ANOVA, for all t-PA responses; $p < 0.001$, ANOVA, for comparisons between saline placebo and B9340 administration for bradykinin infusion (right) only.

3.4.2 PROTOCOL 2: ONSET AND OFFSET OF B9340 ACTION

Subjects were aged 30 ± 2 years with a body mass index of 21 ± 2 kg/m². Baseline blood flow during saline infusion was 3.4 ± 1.0 mL \cdot 100 mL⁻¹ \cdot min⁻¹ in the infused arm and 2.7 ± 0.6 mL \cdot 100 mL⁻¹ \cdot min⁻¹ in the non-infused arm. Bradykinin infusion

caused a $288 \pm 7\%$ increase in the infused FBF that was sustained for 60 minutes (ANOVA, $p < 0.001$; Figure 3.3). Compared with placebo, B9340 coinfusion caused a rapid onset and offset of inhibition of bradykinin-induced vasodilatation (ANOVA, $p < 0.001$; Figure 3.3).

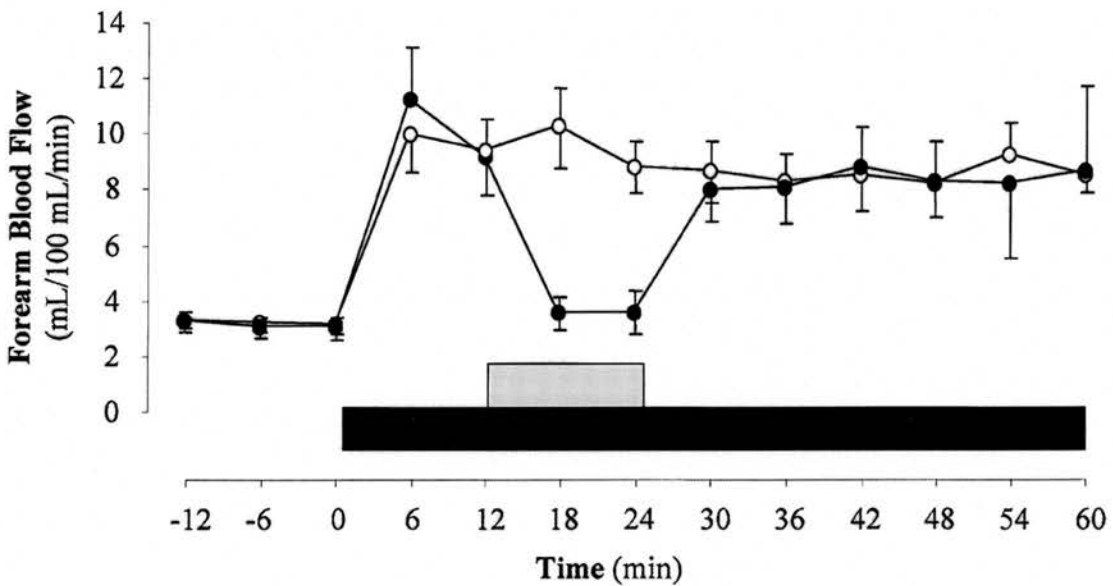


Figure 3.3 Infused arm FBF with bradykinin infusion (black bar; 100 pmol/min) during placebo (open circles) or B9340 (closed circles; 13.5 nmol/min) co-administration (grey bar).

3.5 DISCUSSION

We have confirmed our earlier findings [Labinjoh *et al* 2001; Witherow *et al* 2002] that intrabrachial bradykinin infusion causes marked forearm vasodilatation and endothelial t-PA release. We have demonstrated that B9340 is a potent and competitive antagonist of bradykinin-induced vasodilatation and endothelial t-PA release *in vivo* in humans. B9340 appears to be a selective and reversible bradykinin receptor antagonist with a rapid onset and offset of action.

B9340 caused dose-dependent inhibition of bradykinin-induced vasodilatation and t-PA release, suggesting that it acts as a competitive receptor antagonist and that both vascular effects are mediated through bradykinin receptors. Previous clinical studies [Cockcroft *et al* 1994a; Brown *et al* 2000] have used systemic intravenous administration of the selective B₂ receptor antagonist HOE-140, combined with intrabrachial bradykinin infusions. However, systemic drug administration might have ancillary effects, and the antagonist effects of HOE-140 could have been mediated through intermediary pathways. In contrast, here we have demonstrated a direct, local, dose-dependent inhibition of the vascular actions of bradykinin with intra-arterial infusions of B9340. Moreover, those previous studies of systemic HOE-140 administration either lacked a control vasodilator [Cockcroft *et al* 1994a] or used the endothelium independent vasodilator sodium nitroprusside [Brown *et al* 2000]. We have more rigorously demonstrated the selectivity of bradykinin antagonism by B9340 through comparison with the tachykinin substance P, which acts through the endothelial neurokinin type-1 receptor to cause endothelium-dependent vasodilatation [Newby *et al* 1999a] and t-PA release. The antagonist action of B9340 cannot, therefore, be attributed to a nonspecific effect on the vascular endothelium and appears to be selective for bradykinin receptors.

Bradykinin is thought to exert its vasodilatory effects by activating the B₂ receptor on vascular endothelium. This results in release of nitric oxide [Cockcroft *et al* 1994b; O'Kane *et al* 1994] and endothelium derived hyperpolarising factor to produce vasorelaxation [Beny *et al* 1987; Nakashima *et al* 1993]. However, although inhibition of nitric oxide synthase with N^G-monomethyl-L-arginine attenuates the

vasodilatation to bradykinin, it does not affect endothelial t-PA release [Brown *et al* 2000]. This would suggest that bradykinin stimulated t-PA release is mediated through a nitric oxide synthase independent pathway and that the endothelium regulates blood flow and t-PA release through distinct pathways. Indeed, we have recently demonstrated that direct, local, endothelial t-PA release can be induced by tumour necrosis factor- α . In the absence of alterations in blood flow in the human forearm [Chia *et al* 2003b]. Thus, bradykinin-induced t-PA release appears to be dependent on the endothelial bradykinin receptors and to act through a second-messenger pathway that is distinct from the regulation of vasomotion.

B9340 had no effect on basal FBF or plasma t-PA concentrations, suggesting that in healthy humans, bradykinin does not contribute to the basal maintenance of vascular tone or t-PA release. However, this does not preclude a potential role for bradykinin in pathophysiologic conditions, such as inflammation [Raidoo *et al* 1997], or instances where ACE inhibitor therapy is used [Witherow *et al* 2001; Pretorius *et al* 2003].

Although B9340 antagonises the B₂ receptor at concentrations nearly two orders of magnitude greater than those at the B₁ receptor, B9340 might still produce some blockade of the B₁ receptor. The B₁ receptor was previously thought to be inactive in the vascular system unless upregulated during inflammation. Raidoo *et al* [1997] have recently shown that B₁ receptors are present in large numbers on the vascular endothelium and are further upregulated around atherosclerotic plaques. However, the functional significance of the B₁ receptor on the endothelium is unknown, and the

work by Brown *et al* [2000] using HOE-140 would suggest that the observed effects are mediated principally through blockade of the B₂ receptor.

3.6 CONCLUSIONS

B9340 is a potent, reversible, and selective competitive inhibitor of bradykinin-induced vasodilatation and t-PA release in humans. This compound is a potentially useful investigational tool in dissecting out the physiologic and pathophysiologic role of bradykinin *in vivo* in humans.

CHAPTER 4

**MARKED BRADYKININ-INDUCED TISSUE PLASMINOGEN
ACTIVATOR RELEASE IN PATIENTS WITH HEART FAILURE
MAINTAINED ON CHRONIC ACE INHIBITOR THERAPY**

SUMMARY

Objective - Bradykinin is a potent endothelial cell stimulant that causes vasodilatation and t-PA release. In large scale clinical trials, ACE inhibitor therapy prevents ischaemic events. The aim of the present study was to assess the contribution of ACE inhibitor therapy to bradykinin-induced t-PA release in patients with heart failure secondary to ischaemic heart disease.

Methods and Results - Nine patients with symptomatic heart failure were evaluated on two occasions: during and following 7 day withdrawal of long-term ACE inhibitor therapy. Forearm blood flow was measured using bilateral venous occlusion plethysmography. Intrabrachial bradykinin (30-300 pmol/min), substance P (2-8 pmol/min), and sodium nitroprusside (1-4 pmol/min) were infused, and venous blood samples were withdrawn from both forearms for estimation of fibrinolytic variables. On both study days, bradykinin and substance P caused dose-dependent vasodilatation and release of t-PA from the infused forearm ($p < 0.05$ by ANOVA). Long-term ACE inhibitor therapy caused an increase in forearm vasodilatation ($p < 0.05$ by ANOVA) and t-PA release ($p < 0.001$ by ANOVA) during bradykinin, but not substance P, infusion. Maximal local plasma t-PA activity concentrations approached 100 IU/mL, and maximal forearm protein release was ~ 4.5 $\mu\text{g}/\text{min}$.

Conclusions - Long-term ACE inhibitor therapy augments bradykinin-induced peripheral vasodilatation and local t-PA release in patients with heart failure due to ischaemic heart disease. Local plasma t-PA activity concentrations approached those

seen during systemic thrombolytic therapy for acute myocardial infarction. This may contribute to the primary mechanism of the anti-ischaemic effects associated with long-term ACE inhibitor therapy.

4.2 INTRODUCTION

Bradykinin is a potent endothelium-dependent vasodilator that has a brief duration of action due to its rapid degradation by ACE. In addition to acting as an inflammatory mediator, bradykinin is closely involved in fibrinolytic and coagulation cascades. During the contact phase of blood coagulation, it is released after cleavage of HMW kininogen by kallikrein [Reddigari and Kaplan 1988]. It is also a potent stimulant for the release of t-PA from the endothelium [Labinjoh et al 2001]. Thus, when plaque rupture or erosion activates the intrinsic coagulation pathway, liberation of bradykinin may represent an important negative feedback loop in which bradykinin-induced t-PA release inhibits intravascular thrombus formation.

Large scale clinical trials of patients with heart failure or ischaemic heart disease indicate a reduction in recurrent infarction rates with ACE inhibitor therapy [The Heart Outcomes Prevention Evaluation Study Investigators 2000]. The mechanisms underlying this anti-ischaemic benefit may relate, in part, to the effects on endogenous fibrinolysis. Inhibition of ACE enhances bradykinin-induced vasodilatation and endothelial t-PA release in healthy volunteers [Labinjoh *et al* 2001]. However, to date, there has been no assessment of the effect of long-term ACE inhibition on acute endogenous t-PA release in patients with heart failure or ischaemic heart disease. Therefore, the aim of this study was to determine whether long-term ACE inhibition potentiates acute t-PA release in patients with heart failure secondary to ischaemic heart disease.

4.3 METHODS

4.3.1 STUDY SUBJECTS

Nine patients with New York Heart Association (NYHA) functional class II or III heart failure secondary to ischaemic heart disease participated in the study, which was undertaken with the approval of the Local Research Ethics Committee, in accordance with the Declaration of Helsinki (1989), and each subject gave written, informed consent. All subjects had been maintained on a maximally tolerated dose of an ACE inhibitor for more than 6 months, and they abstained from alcohol for 24 hours and from food and caffeine containing drinks for at least 4 hours before each study.

4.3.2 MEASUREMENTS

Forearm blood flow and haemodynamics

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury in silastic strain gauges applied to the widest part of the forearm, as previously described [Newby *et al* 1997; Labinjoh *et al* 2001]. Blood pressure and heart rate were monitored in the non-infused arm at intervals throughout each study by using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc., Tokyo, Japan).

Assays

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossa in both arms. Ten to twenty millilitres of blood were withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool Stabilyte, Umeå, Sweden; for t-PA assays) and citrate (Monovette, Sarstedt, Nümbrecht, Germany; for PAI-1 assays) tubes and kept on ice before being centrifuged at 2,000 g for 30 minutes at 4°C. Platelet free plasma was decanted and stored at -80°C before assay. Plasma t-PA and PAI-1 antigen and activity concentrations were determined using enzyme-linked immunosorbent assays and a photometric method, as previously described [Newby *et al* 1997; Labinjoh *et al* 2001].

4.3.3 STUDY DESIGN

Patients were evaluated at 9 a.m. on two occasions: during and after 7 day withdrawal of long-term ACE inhibitor therapy. On the appropriate study day, oral ACE inhibitor therapy was administered at 8 a.m. The brachial artery of the non-dominant arm was cannulated with a standard 27-gauge steel wire needle (Cooper's Needle Works Ltd., Birmingham, UK) under local anaesthesia. The total rate of intra-arterial infusions was maintained constant at 1 mL/min, and FBF was measured every 10 minutes throughout all studies. Intrabrachial infusions of substance P (Clinalfa AG, Läufelfingen, Switzerland) at 2, 4, and 8 pmol/min, bradykinin (Clinalfa AG) at 30, 100, and 300 pmol/min, and sodium nitroprusside (David Bull Laboratories, Warwick, UK) were given at 1, 2, and 4 µg/min for 10 minutes at each

dose in a randomised order [Labinjoh *et al* 2001]. Saline was infused for 30 minutes before the substance P, sodium nitroprusside, and bradykinin infusions.

4.3.4 DATA ANALYSIS AND STATISTICS

Forearm blood flow was calculated for individual venous occlusion cuff inflations, as previously described [Newby *et al* 1997; Labinjoh *et al* 2001]. Estimated net release of t-PA activity and antigen was previously defined as the product of the infused FBF (based on the mean haematocrit and infused FBF) and the concentration difference between the infused and non-infused arms [Newby *et al* 1997; Labinjoh *et al* 2001]. Data were examined, where appropriate, by ANOVA with repeated measures and the 2-tailed paired Student *t*-test, using Microsoft Excel 97. All results are expressed as the mean value \pm SEM. Statistical significance was set at the 5% level.

4.4 RESULTS

Patient characteristics are shown in Table 4.1. After withdrawal of ACE inhibitor therapy, baseline mean arterial pressure appeared to rise, but this was not statistically significant. There were no significant changes in heart rate, blood pressure or non-infused FBF (Table 4.1, Figure 4.1) during or between the study days.

Number	9
Age	65(53-79)
Sex, male:female	9:0
Medication	
ACE inhibition	9
Aspirin	8
Diuretic	6
B-Blocker	2
Statin	8
Nitrate	4
Digoxin	1
Calcium Antagonist	1
Spironolactone	1
Warfarin	3
EF (%)	25.1±4.6
SF (%)	9.7±2.1
LVEDD (mm)	6.4±0.2
Heart Rate (beats/min)	
Visit 1	61.1±4.7
Visit 2	60.8±3.7
MAP (mmHg)*	
Visit 1	83.9±3.5
Visit 2	88.6±5.4
Basal FBF (mL/100mL per min)	
Visit1	
Non-infused	2.3±0.2
Infused	2.3±0.2
Visit2	
Non-infused	2.8±0.6
Infused	2.6±0.3

Table 4.1 Patient characteristics (n = 9). Data are presented as the number of patients or mean value ± SEM. *Diastolic pressure + one-third of pulse pressure. ACE - angiotensin-converting enzyme; LVEDD - left ventricular end diastolic diameter; Visit 1 - during long-term ACE inhibition; Visit 2 - after 7 day withdrawal of long-term ACE inhibition.

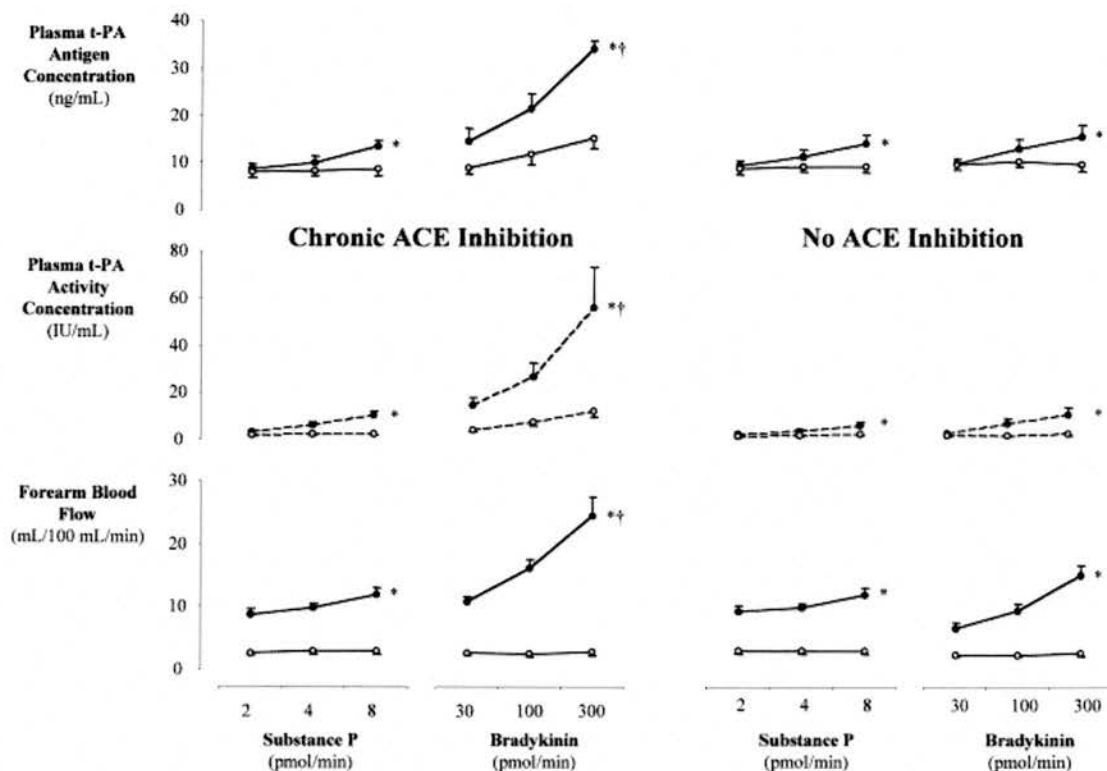


Figure 4.1 Effect of intra-arterial bradykinin and substance P infusions on blood flow and plasma tissue-type plasminogen activator (t-PA) antigen (solid lines) and activity (dashed lines) concentrations in the infused (solid symbols) and non-infused (open symbols) arms in the presence (left) or absence (right) of angiotensin-converting enzyme (ACE) inhibition. * $p < 0.05$ by analysis of variance (ANOVA). † $p < 0.001$ by ANOVA for long-term ACE inhibition *versus* no ACE inhibition.

4.4.1 FOREARM BLOOD FLOW RESPONSES

Sodium nitroprusside (data on file), substance P, and bradykinin caused dose-dependent forearm vasodilatation during each study visit (ANOVA for blood flow response, $p < 0.001$ for all; $n = 9$ at baseline and with the three doses, degree of freedom [d.f.] = 3) (Figure 4.1). In the presence of long-term ACE inhibition, forearm vasodilatation was augmented during bradykinin (ANOVA for ACE inhibition *versus* no ACE inhibition, $p < 0.05$; $n = 9$ for blood flow at baseline and with the three doses, d.f. = 1), but not substance P or sodium nitroprusside ($p = \text{NS}$) infusion.

4.4.2 PLASMA FIBRINOLYTIC VARIABLES

Bradykinin and substance P caused dose-dependent increases in plasma t-PA antigen and activity concentrations in the infused arm (ANOVA for plasma t-PA concentrations, $p < 0.05$ for all; $n = 9$ at baseline and with the three doses, $d.f. = 3$) (Figure 4.1). Plasma t-PA antigen and activity concentrations were significantly augmented during bradykinin (ANOVA for ACE inhibition *versus* no ACE inhibition, $p < 0.001$; $n = 9$ for plasma t-PA concentrations at baseline and with the three doses, $d.f. = 1$), but not substance P ($p = NS$) infusion in the presence of long-term ACE inhibition.

Basal plasma PAI-1 antigen concentrations were lower in the presence (38 ± 6 ng/mL) than in the absence (48 ± 7 ng/mL) of ACE inhibitor therapy (paired *t*-test for ACE inhibition *versus* no ACE inhibition, $p < 0.02$; $n = 9$ for plasma PAI-1 concentrations, $d.f. = 1$). Bradykinin and substance P administration had no effect on plasma PAI-1 concentrations.

4.4.3 RELEASE OF TISSUE PLASMINOGEN ACTIVATOR

Substance P and bradykinin caused dose-dependent increases in the plasma t-PA antigen and activity concentration differences between the forearms, as well as the estimated net release of t-PA antigen and activity during each study visit (ANOVA for t-PA release, $p < 0.01$ for all; $n = 9$ for t-PA release or concentration difference at baseline and with the three doses, $d.f. = 3$) (Figure 4.2). During ACE inhibition, there was a massive increase in bradykinin-induced (ANOVA for ACE inhibition *versus* no ACE inhibition, $p < 0.001$; $n = 9$ for t-PA release at baseline and with the three

doses, d.f.=1) (Figure 4.2), but not substance P induced ($p=NS$), release of t-PA antigen and activity (increases in the area under the curve of 520% and 877%, respectively). Post-hoc analysis identified no significant effect of other concomitant medications on substance P-induced or bradykinin-induced t-PA release.

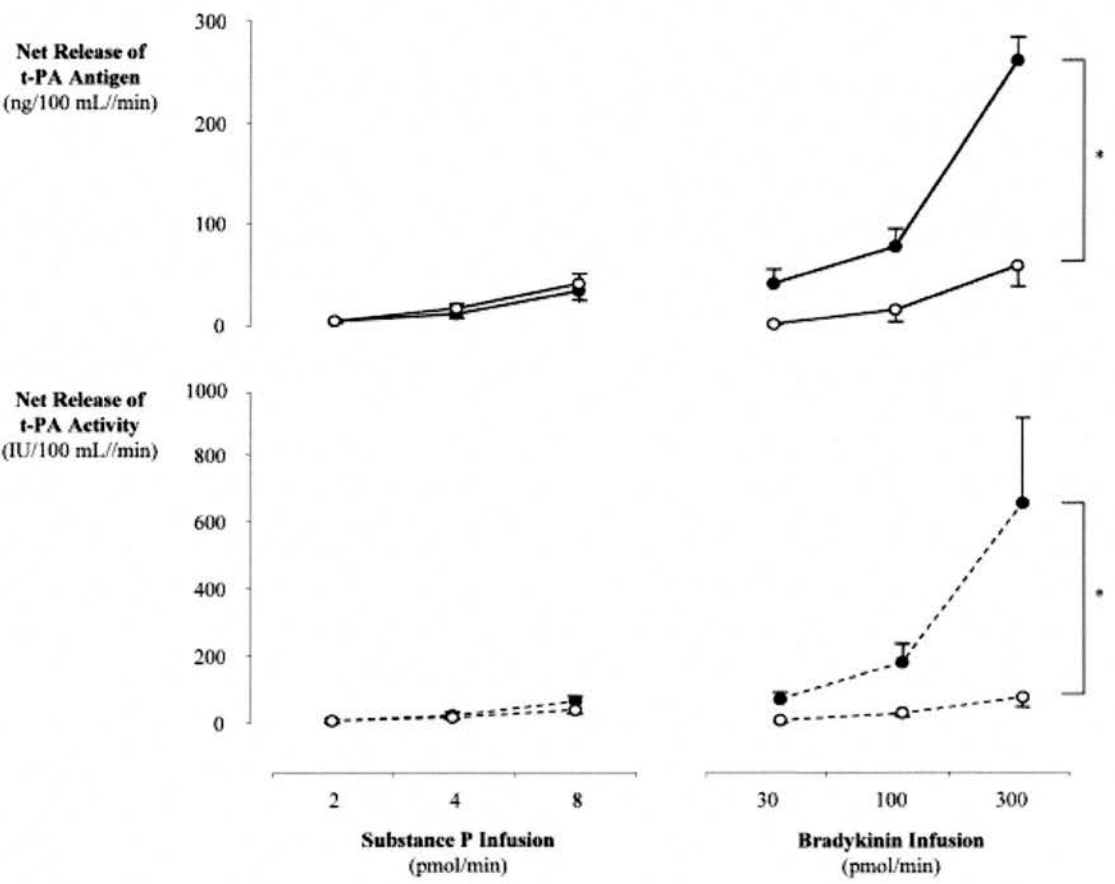


Figure 4.2 Estimated net release of tissue-type plasminogen activator (t-PA) antigen (solid lines) and activity (dashed lines) during bradykinin (right) and substance P (left) infusions in the presence (solid circles) or absence (open circles) of angiotensin-converting enzyme (ACE) inhibition. $p < 0.01$ by analysis of variance (ANOVA) for all responses. * $p < 0.001$ by ANOVA for long-term ACE inhibition *versus* no ACE inhibition.

4.5 DISCUSSION

For the first time, to the best of our knowledge, we have shown that in patients with heart failure secondary to ischaemic heart disease, long-term ACE inhibition markedly potentiates bradykinin-induced endogenous t-PA release from the endothelium. However, this potentiation appears to be specific to bradykinin, because ACE inhibition did not influence substance P-induced t-PA release. These findings suggest that the beneficial clinical and vascular effects of ACE inhibition may be partly mediated through the acute local augmentation of bradykinin-induced t-PA release.

4.5.1 MAGNITUDE OF t-PA RELEASE

Long-term ACE inhibition produced a massive augmentation of bradykinin-induced t-PA release across the forearm vascular bed. Although these doses of bradykinin are supra physiological, the approximate 5-fold increase in t-PA antigen release and the ~20% reduction in plasma PAI-1 antigen concentrations led to the approximate 9-fold increase in the release of active t-PA. Our group has previously shown that bradykinin-induced t-PA release is augmented by ACE inhibition in healthy volunteers, but this was modest at approximately 2-fold only [Labinjoh *et al* 2001]. The dramatic potentiation of active t-PA release in the present study is exemplified by the observation that the maximal local forearm concentrations of active t-PA (99 IU/mL at 300 pmol/min of bradykinin) approached those observed during systemic thrombolysis during acute myocardial infarction (100 to 1,000 IU/mL) [Koster *et al* 1991]. Moreover, it also underscores the large capacity of the endothelium to release

t-PA quickly, up to 4.5 μ g or 16,000 IU/min from the infused forearm at 300 pmol/min of bradykinin. Indeed, using intrabrachial substance P infusions, we have previously demonstrated substantial and sustained release of t-PA for up to 2 hours [Newby *et al* 1998].

Minai *et al* [2001] have recently reported that ACE inhibition produces an approximate 2-fold increase in bradykinin-induced t-PA release in the coronary circulation of patients with atypical chest pain and angiographically normal coronary arteries. This is consistent with our previous findings in the peripheral circulation of healthy volunteers [Labinjoh *et al* 2001] and suggests that comparable endothelial fibrinolytic effects exist between the peripheral and coronary circulations [Newby *et al* 2001b]. The present study extends these previous findings, because we have demonstrated a more marked augmentation of peripheral t-PA release in patients with heart failure secondary to ischaemic heart disease.

4.5.2 MECHANISM OF BRADYKININ-INDUCED T-PA RELEASE

In keeping with our previous work [Labinjoh *et al* 2001] and that by others [Benjamin *et al* 1989], ACE inhibition augmented the vasodilatation induced by bradykinin but did not affect the vasodilatation or t-PA release produced by substance P. This suggests that the effect of ACE inhibition does not appear to reflect a generalised enhancement of vascular function but is specific to bradykinin. Brown *et al* [2000] and Gainer *et al* [2001] have previously investigated the mechanism of bradykinin-induced t-PA release in the human forearm. Bradykinin induces t-PA release through a B₂ receptor dependent, nitric oxide synthase independent, and

cyclooxygenase independent pathway [Brown *et al* 2000]. Brown *et al* [2000] have suggested that bradykinin-induced t-PA release may be caused by an endothelium-derived hyperpolarising factor, although other mediators may be involved. This group has also described a potential interaction between the vascular responses to bradykinin and the ACE gene insertion/deletion polymorphism [Gainer *et al* 2001]. We have not explored this interaction because of the small sample size of our study, but this may markedly influence the fibrinolytic response to long-term ACE inhibition in patients with heart failure or vascular disease, and it requires further investigation.

Inflammation plays an important role in the pathogenesis of heart failure [Gottdiener *et al* 2000], with elevated plasma concentrations of circulating cytokines such as tumour necrosis factor- α [Feldman *et al* 2000]. Bradykinin receptor expression is altered by ACE inhibition [Miyamoto *et al* 2000; Marin-Castano *et al* 2002], inflammation [Marceau 1995], and chronic heart failure [Witherow *et al* 2001]. ACE inhibitor therapy alters the B2 receptor to have high and low affinity sites for bradykinin binding, and B1 receptor expression is upregulated in arteries by ACE inhibition and these findings may partly explain the proportionately greater and massive release of t-PA from the endothelium [Miyamoto *et al* 2000; Marin-Castano *et al* 2002].

4.5.3 ENDOTHELIAL FUNCTION, ENDOGENOUS FIBRINOLYSIS, AND HEART FAILURE

The endothelium plays a vital role in the control of blood flow, haemostasis, fibrinolysis, and inflammation. Consequently, the maintenance and regulation of

tissue perfusion critically depends on the integrity of endothelial function and the release of potent endothelium-derived factors. After the seminal work of Furchgott and Zawadski [1980], it has been widely recognised that an array of mediators can influence vascular tone through endothelium-dependent actions, and there is now extensive evidence of abnormal endothelium-dependent vasomotion that is reversed by ACE inhibition in patients with heart failure [Drexler *et al* 1995; Varin *et al* 2000]. However, although endothelium-dependent vasomotion is important, it may not be representative of other aspects of endothelial function, such as the regulation of endogenous fibrinolysis.

Tissue plasminogen activator is a serine protease that regulates the degradation of intravascular fibrin and is released from the endothelium through the translocation of a dynamic intracellular storage pool. If endogenous fibrinolysis is to be effective, then the rapid mobilisation of t-PA from the endothelium is essential, because thrombus dissolution is much more effective if t-PA is incorporated during, rather than after, thrombus formation [Fox *et al* 1984]. This dynamic aspect of endothelial function and fibrinolytic balance may be directly relevant to the pathogenesis of atherothrombosis and is not necessarily reflected by the basal plasma concentrations of t-PA [Jern *et al* 1999; Newby *et al* 1999; Newby *et al* 2001a].

4.5.4 CLINICAL RELEVANCE

The endogenous fibrinolytic system can have important clinical effects, as exemplified by the observation that in one-third of patients with an acute myocardial infarction, the infarct related artery spontaneously reperfuses within 12 hours

[DeWood *et al* 1980; Armstrong *et al* 1989; Rentrop *et al* 1989]. Moreover, low fibrinolytic activity is associated with an increased risk of in young men [Meade *et al* 1993] and predicts which patients with unstable angina will develop myocardial infarction [Munkvad *et al* 1990]. Clinical studies of patients with unstable angina have also indicated that there is an enhanced activation of the kallikrein system and that bradykinin release is increased [Hoffmeister *et al* 1995]. Given this augmentation of bradykinin generation and activation of the intrinsic coagulation pathway in acute coronary syndromes, ACE inhibition may have major beneficial effects on the acute local fibrinolytic balance by markedly enhancing bradykinin-induced t-PA release in areas of intravascular thrombus formation. This is consistent with the observation that ACE inhibition improves the basal fibrinolytic balance [Wright *et al* 1994; Vaughan *et al* 1995b] and reduces myocardial troponin release in patients with acute coronary syndromes [Kennon *et al* 2001].

4.6 CONCLUSIONS

Long-term angiotensin-converting enzyme inhibitor therapy augments bradykinin-induced peripheral vasodilatation and local t-PA release in patients with heart failure due to ischaemic heart disease. Local plasma t-PA activity concentrations approached those seen during systemic thrombolytic therapy for acute myocardial infarction. This may contribute to the primary mechanism of the anti-ischaemic effects associated with long-term ACE inhibitor therapy.

CHAPTER 5

BRADYKININ CONTRIBUTES TO THE VASODILATOR EFFECTS OF CHRONIC ACE INHIBITION IN PATIENTS WITH HEART FAILURE

5.1 SUMMARY

Background - Bradykinin, an endogenous vasodilator peptide, is metabolised by ACE. The aims of the present study were to determine the doses of B9340, a bradykinin receptor antagonist, that inhibit vasodilatation to exogenous bradykinin and to assess the contribution of bradykinin to the maintenance of basal vascular tone in patients with heart failure receiving chronic ACE inhibitor therapy.

Methods and Results - Forearm blood flow was measured using bilateral venous occlusion plethysmography. On three occasions in a double-blind randomised manner, eight healthy volunteers received intrabrachial infusions of placebo or B9340 (at 4.5 and 13.5 nmol/min). On each occasion, placebo or B9340 was coinjected with bradykinin (30-3,000 pmol/min) and substance P (4-16 pmol/min). B9340 caused no change in basal FBF but produced dose-dependent inhibition of the vasodilatation to bradykinin ($p < 0.001$) but not substance P. The effects of bradykinin antagonism were studied in 17 patients with NYHA grade II-IV heart failure maintained on chronic ACE inhibitor therapy. Incremental doses of B9340, but not HOE-140, produced a dose-dependent vasoconstriction ($p < 0.01$). After withdrawal of ACE inhibitor therapy, B9340 produced no significant change in FBF. After reinstitution of therapy, B9340 again resulted in vasoconstriction ($p < 0.03$).

Conclusions - B9340 is a potent and selective inhibitor of bradykinin-induced vasodilatation. Bradykinin does not contribute to the maintenance of basal peripheral arteriolar tone in healthy humans or patients with heart failure but contributes to the vasodilatation associated with chronic ACE inhibitor therapy in patients with heart failure via the B_1 receptor.

5.2 INTRODUCTION

Patients with heart failure have a reduced cardiac reserve that is associated with neurohumoral activation of the renin-angiotensin-aldosterone system and peripheral vasoconstriction. Through blockade of angiotensin I conversion, ACE inhibitor therapy limits the generation of angiotensin II, thereby reducing the associated vasoconstriction and sodium and water retention. It has been widely established that ACE inhibitor therapy has major therapeutic benefits in patients with heart failure [Cohn *et al* 1991; The AIRE investigators 1993], which include improvements in morbidity, exercise capacity, and mortality (The CONSENSUS Trial Study Group 1987; Cohn *et al* 1991). The administration of ACE inhibitor therapy in these patients causes systemic vasodilatation [Flapan *et al* 1992] that has been attributed to the loss of angiotensin II mediated vasoconstriction. However, it is unknown whether the vasodilatation associated with ACE inhibitor therapy may in part relate to the concomitant blockade of bradykinin degradation.

Bradykinin is a potent endothelium-dependent vasodilator [(Cherry *et al* 1982; Cockcroft *et al* 1994)] that has a brief duration of action (plasma half-life of 15-30 seconds) because of its rapid degradation by ACE. Indeed, ACE breaks down >95% of bradykinin in a single passage through the pulmonary circulation [Bonner *et al* 1992]. Exogenous administration of bradykinin induces vasodilatation of epicardial coronary [Groves *et al* 1995; Kuga *et al* 1997] and resistance arteries in humans, which is mediated in part by nitric oxide [O'Kane *et al* 1994] and endothelium-derived hyperpolarising factor [Honing *et al* 2000]. The local and

systemic vascular effects of exogenous bradykinin administration can be enhanced by ACE inhibition [Benjamin *et al* 1989; Bonner *et al* 1992]. In hypertensive patients and sodium deplete volunteers, Gainer *et al* [1998] have shown that systemic infusion of HOE-140 (icatibant), a highly selective bradykinin B₂ receptor antagonist, attenuated the hypotensive effects of captopril and resulted in a similar reduction in blood pressure to losartan, an angiotensin II type-1 receptor blocker. This suggests that some of the short-term hypotensive effects of ACE inhibition are mediated by augmentation of endogenous bradykinin. This study has, however, been criticised for its design, because the observed pressor effect may have been attributable to the differing pharmacokinetic profiles of losartan and captopril [Azizi 1999]. Moreover, it did not address other pertinent issues, such as the effect of chronic ACE inhibition, or the potential role of the B₁ receptor.

When examining *in vivo* vascular responses in humans, systemic drug administration causes concomitant effects on organs, such as the brain, kidney, and heart, and influences neurohumoral reflexes through changes in systemic haemodynamic parameters. Because of these influences, vascular responses cannot be wholly attributed to a direct effect of the drug on blood vessels [Benjamin *et al* 1989; Webb 1995]. In contrast, the use of bilateral FBF measurements, with unilateral brachial artery infusion of vasoactive drugs at sub systemic, locally active doses, provides a powerful and reproducible method of directly assessing vascular responses *in vivo*. This technique has been used to demonstrate the major contribution of nitric oxide and endothelin-1 to the maintenance of basal peripheral vascular tone [Valance *et al* 1989; Haynes and Webb 1994] and to predict the pressor and depressor effects of

systemic nitric oxide inhibition and endothelin receptor antagonism, respectively [Haynes *et al* 1993; Haynes *et al* 1996].

Several peptidic and nonpeptidic bradykinin receptor antagonists are presently under development. However, to date, there has been no assessment of the combined contribution of B₁ and B₂ receptors to basal vascular tone in patients with heart failure maintained on chronic ACE inhibitor therapy. B9340, a peptidic analogue of HOE-140, has inhibitory activity against both B₁ and B₂ receptors [Stewart *et al* 1997] and has recently become available for use in clinical studies. Therefore, the aims of the present study were, first, to confirm that B9340 is a potent and specific bradykinin receptor antagonist *in vivo* in humans; second, to establish the role of bradykinin in the maintenance of peripheral vascular tone in healthy volunteers; and, third, to determine whether endogenous bradykinin contributes to the maintenance of basal peripheral vascular tone in patients with heart failure maintained on chronic ACE inhibitor therapy using both the selective and nonselective bradykinin receptor antagonists, HOE-140 and B9340, respectively.

5.3 METHODS

5.3.1 STUDY SUBJECTS

The protocols were performed with the approval of the Local Research Ethics Committee in accordance with the Declaration of Helsinki (1989) and with the written informed consent of each subject. As in Chapter 3 the same eight healthy male non smokers attended on three separate occasions at least 1 week apart. Each

volunteer was studied at the same time of day and was fasting for at least 4 hours and avoided alcohol and caffeine for 24 hours before each study. No medications or vasoactive drugs were taken in the 7 days before the study.

Seventeen patients with stable NYHA grade II-IV heart failure, established for at least 6 months on maximally tolerated ACE inhibitor therapy, were enrolled in the study. The patients attended fasted before each study, and diuretics were withheld on the morning of the study for patient comfort. Twelve patients initially attended at 9.00 a.m. on two occasions 1 week apart. ACE inhibitor therapy was withheld for 1 week before the second visit and recommenced after the second study. Nine patients returned at least 4 weeks after recommencing their ACE inhibitor therapy and underwent a repeat study. Two patients withdrew their consent because of personal reasons, and one patient changed therapy before the third visit and was thus excluded. Nine patients, including four patients who attended on the first three occasions, were subsequently studied on a final occasion.

5.3.2 MEASUREMENTS

Intra-arterial drug administration and FBF measurements were performed as previously described [Webb 1995; Newby *et al* 1999b]. Heart rate and blood pressure were measured non-invasively in the non-infused arm immediately after the FBF measurements every 10 minutes throughout each study using a semi-automated, sphygmomanometer (UA-731, A&D Engineering).

5.3.3 DRUGS

B9340 (molecular weight 1318.6) is a synthetic peptide antagonist of bradykinin that differs from HOE-140 (icatibant; molecular weight 1304.6) by replacement of the α -(2-indanyl)glycine at position 7 of the molecule with a tetrahydroisoquiniline-3-carboxylic acid moiety. When compared with HOE-140 in animal studies, B9340 retains similar potency of inhibition at the B₂ receptor (pIC₅₀ of 9.8 for both) but produces more than a 100-fold greater inhibition at the B₁ receptor (pIC₅₀ of 6.0 and 8.1 for HOE-140 and B9340, respectively) [Stewart *et al* 1997]. Pharmaceutical grade B9340 (Clinalfa AG), HOE-140 (Clinalfa), substance P (Clinalfa), and bradykinin (Clinalfa) were dissolved in 0.9% saline before infusion. All solutions were freshly prepared on the day of study.

5.3.4 PROTOCOL DESIGN

Healthy Volunteer Study

After 30 minutes equilibration with saline infusion, intra-arterial placebo (0.9% saline), B9340 at 4.5 nmol/min, or B9340 at 13.5 nmol/min was infused for 100 minutes on separate occasions in a randomised, ascending dose, double-blind manner. Placebo or B9340 was coinjected with saline, with bradykinin at 30, 300, and 3000 pmol/min for 10 minutes at each dose, and, after 30 minutes saline infusion, with substance P at 4, 8, and 16 pmol/min for 10 minutes at each dose. Throughout the study, FBF was measured every 10 minutes, and the final FBF measurement taken during saline infusion was defined as the baseline FBF.

Patient Study

On each of the first three occasions, B9340 was infused intra-arterially at 0.45, 1.35, 4.5, and 13.5 nmol/min for 6 minutes at each dose. The two extra doses were included to demonstrate dose response. Subsequently, on the final occasion in nine patients, HOE-140 was infused intra-arterially at 0.45, 1.35, 4.5, and 13.5 nmol/min for 6 minutes at each dose. This dose was chosen to match that of B9340 which has similar potency at the B2 receptor. On each occasion, drug infusion was preceded by a 30 minute saline infusion, and unblinded FBF measurements were made for the last 3 minutes of each infusion period.

5.3.5 DATA ANALYSIS AND STATISTICS

Mean arterial pressure was defined as the diastolic blood pressure plus a third of the pulse pressure. Plethysmographic data were extracted from the Chart data files, and FBF was calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 97, Microsoft). Recordings from the first 60 seconds after wrist cuff inflation were not used because of the variability in blood flow that this causes [Webb 1995]. Usually the last five flow recordings in each 3 minute measurement period were calculated and averaged for each arm. To reduce the variability of the blood flow data, the ratio of flows in the two arms was calculated for each time point; in effect using the non-infused arm as a contemporaneous control [Petrie *et al* 2000]. The percentage change in FBF after drug administration was calculated as follows:

$$100 \times \frac{F(i)_d/F(ni)_d - F(i)_v/F(ni)_v}{F(i)_v/F(ni)_v} \%$$

where $F(i)$ and $F(ni)$ represent measured blood flows in the infused and non-infused arms, respectively, during periods of drug (d) and vehicle (v) administration [Webb *et al* 1995].

Data are presented as mean \pm SEM. On the basis of the responses, dose response shifts were calculated for the ED_{300} , the dose producing a 300% increase in basal FBF. Comparisons between groups were made using 2-way ANOVA and, where appropriate, the paired Student's *t*-test. Statistical significance was taken at the 5% level.

5.4 RESULTS

Healthy Volunteer Study

Mean age of the subjects was 25 ± 5 years, and mean body mass index was 22 ± 2 kg/m². B9340 was well tolerated without any adverse events. There were no significant changes in FBF in the non-infused arm, heart rate, or mean arterial pressure during the infusions (data on file). B9340 infusion alone caused no significant change in infused FBF at either dose (at 4.5 nmol/min, $8 \pm 8\%$; at 13.5 nmol/min, $4 \pm 5\%$) compared with placebo ($-7 \pm 7\%$; 2-way ANOVA, placebo *versus* high dose, $p=0.8$).

During placebo infusion, both bradykinin and substance P caused dose-dependent vasodilatation in the infused forearm (ANOVA, $p<0.001$ for both). At doses of 4.5 and 13.5 nmol/min, B9340 inhibited the vasodilatation produced by intra-arterial bradykinin (2-way ANOVA, $p<0.001$ for both doses; Figure 5.1) and resulted in a

5-fold and 30-fold increase in the ED₃₀₀ of bradykinin respectively. B9340 infusion had no effect on substance P-induced vasodilatation (Figure 5.1).

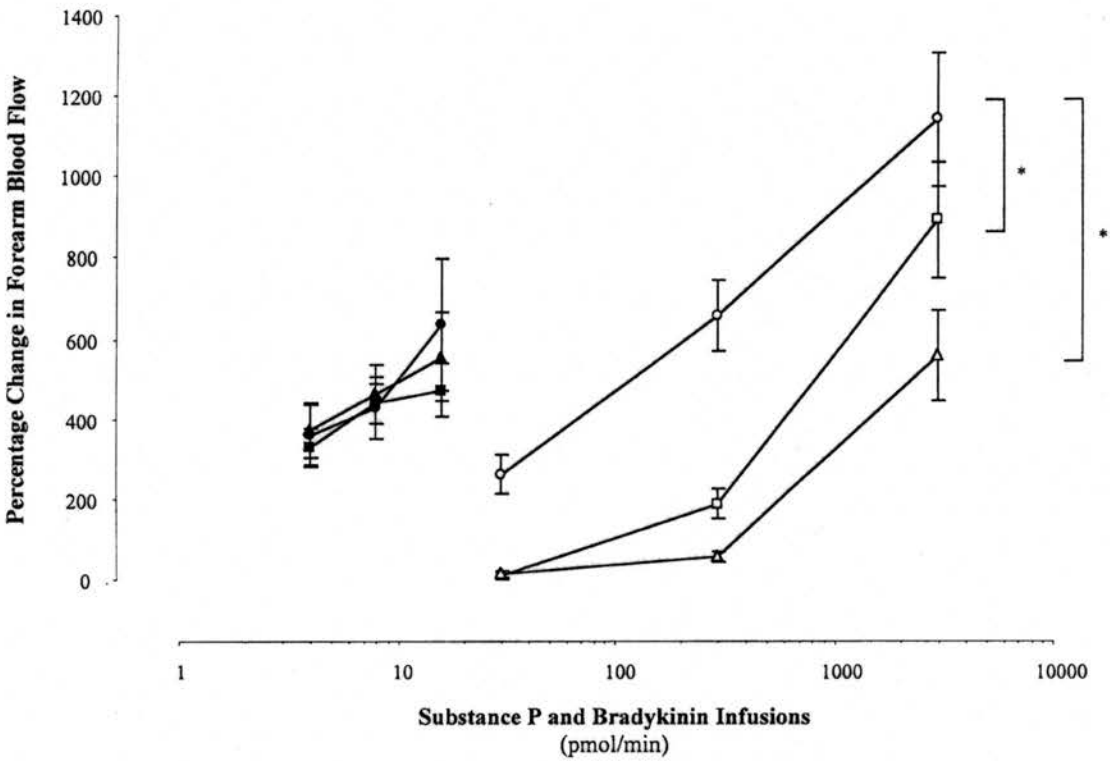


Figure 5.1 Percentage change in forearm blood flow during substance P (closed symbols) and bradykinin (open symbols) infusion during coinfusion of placebo (circles), B9340 at 4.5 nmol/min (squares), and B9340 at 13.5 nmol/min (triangles) in healthy volunteers. * $p < 0.001$. 2-way ANOVA with repeated measures.

Patient Study

Patient characteristics are shown in Table 5.1. Two patients reported an increase in symptoms of breathlessness after withdrawal of their ACE inhibitor therapy for 1 week, which improved after recommencing therapy. During B9340 infusion, there were no significant changes in heart rate, mean arterial pressure, or baseline FBF between the 2 study days, although there was a trend for mean arterial pressure to be

higher when withdrawn from ACE inhibitor therapy (99 ± 4 mmHg *versus* 93 ± 4 mmHg, paired *t*-test, $p=0.08$).

	Visit 1	Visit 2	Visit 3	Visit 4
Number	12	...	9	9
Age (range), years	65 (51-78)	...	65 (52-78)	65(51-79)
Sex, male:female	10:2	...	7:2	9:0
NYHA grade, II:III:IV	4:6:2	...	2:6:1	5:4:0
IHD:DCM	9:3	...	6:3	8:1
Medication				
ACE inhibition	12	...	9	9
Aspirin	11	...	8	6
Diuretic	10	...	7	7
β -Blocker	3	...	2	2
Lipid-lowering therapy	6	...	5	5
Nitrate	5	...	4	3
Digoxin	5	...	4	2
Calcium antagonist	3	...	3	3
Amiodarone	2	...	2	2
Warfarin	1	...	1	3
EF, %	36 ± 5	...	36 ± 6	27 ± 4
SF, %	14 ± 3	...	15 ± 3	10 ± 4
LVEDD, mm	71 ± 4	...	68 ± 4	68 ± 5
Heart rate, beats/min	60 ± 4	55 ± 4	55 ± 4	67 ± 4
MAP, mm Hg	93 ± 4	99 ± 4	108 ± 8	91 ± 5
Basal FBF, mL/100 mL per min				
Infused	2.8 ± 0.3	2.7 ± 0.3	2.8 ± 0.3	2.9 ± 0.4
Non-infused	2.6 ± 0.2	3.5 ± 0.5	2.6 ± 0.3	2.6 ± 0.3

Table 5.1 Patient Characteristics. Values are mean \pm SEM, mean (range), or number of patients. DCM indicates dilated cardiomyopathy; EF, ejection fraction; FBF, forearm blood flow; IHD, ischaemic heart disease; LVEDD, left ventricular end diastolic diameter; MAP, mean arterial pressure; and SF, shortening fraction.

B9340 caused a dose-dependent vasoconstriction in the infused forearm during chronic ACE inhibition (ANOVA, $p=0.01$; Figure 5.2) that was abolished after withdrawal of therapy (2-way ANOVA, ACE inhibition *versus* no ACE inhibition, $p<0.001$). Moreover, B9340 again caused a dose-dependent vasoconstriction (ANOVA, $p<0.03$) in the nine patients who returned for an additional visit at least 4 weeks after recommencing maintenance ACE inhibitor therapy. The change in FBF was not significantly different between the first and third visits (2-way ANOVA, $p=NS$), but it was again significantly different from the response after withdrawal of ACE inhibitor therapy (2-way ANOVA, $p<0.005$). On the final occasion, intra-arterial infusion of HOE-140 had no significant effect on basal FBF during maintenance ACE inhibitor therapy (ANOVA, $p=NS$; Figure 5.2).

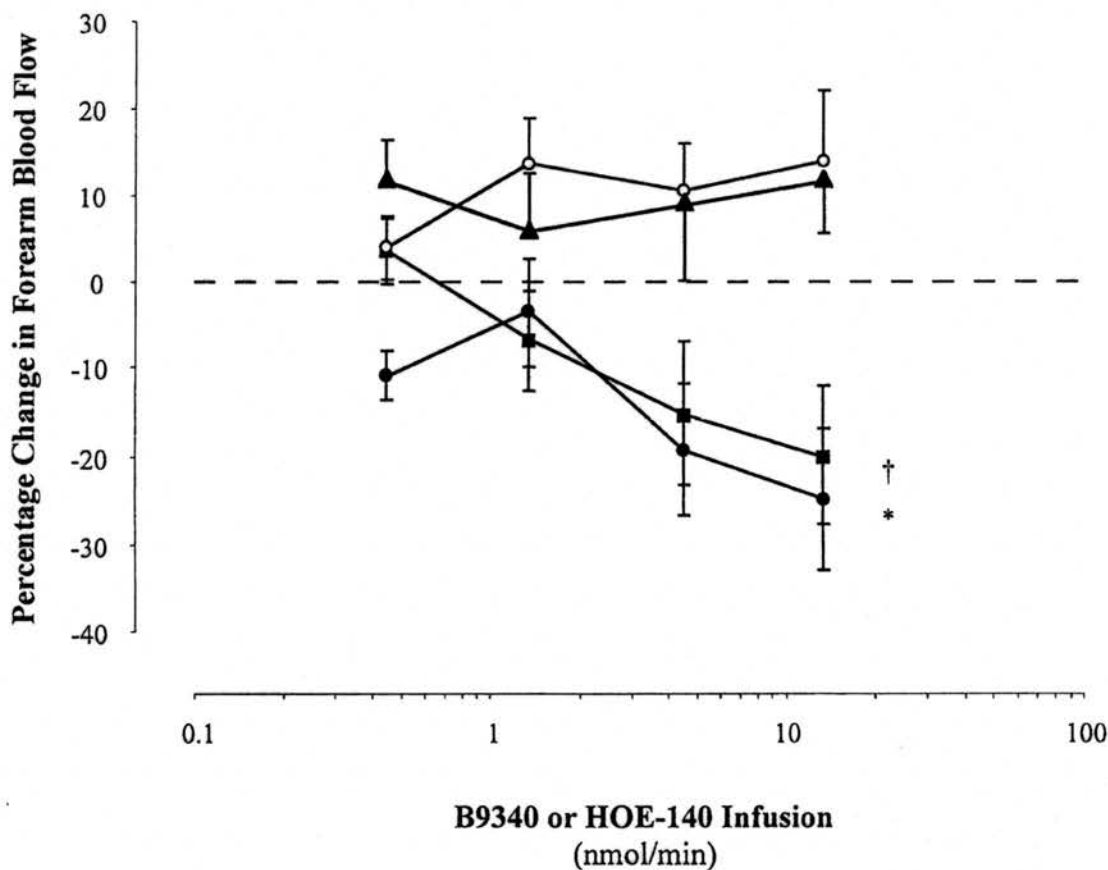


Figure 5.2 Percentage change in forearm blood flow with intrabrachial infusion of B9340 during ACE inhibition (●, $n=12$), after withdrawal of ACE inhibition (○, $n=12$), and after reinstitution of therapy (■, $n=9$) in patients with heart failure. Subsequent forearm blood flow response to intrabrachial HOE-140 infusion in patients with heart failure maintained on ACE inhibitor therapy is also shown (▲, $n=9$). There were no significant changes in forearm blood flow with B9340 in the absence of ACE inhibition or HOE-140 in the presence of ACE inhibition. * $p<0.01$; † $p<0.03$. ANOVA with repeated measures.

5.5 DISCUSSION

We have shown that B9340, a novel peptidic bradykinin receptor antagonist, causes a dose-dependent inhibition of bradykinin mediated forearm vasodilatation without influencing responses to substance P or affecting basal vascular tone. In addition, we have shown for the first time that B9340 causes peripheral vasoconstriction in patients with heart failure during the maintenance of chronic ACE inhibitor therapy. We conclude that in patients with heart failure, endogenous bradykinin may contribute to the vasodilatation produced by chronic ACE inhibition.

In healthy volunteers, we have shown that B9340 selectively inhibits vasodilatation mediated by bradykinin but not substance P. Moreover, when B9340 is infused into the forearm at doses sufficient to increase the ED₃₀₀ of bradykinin 30-fold, it causes no significant change in basal FBF. This indicates that bradykinin, unlike nitric oxide, does not provide a major contribution to the regulation of basal vascular tone in healthy humans. This is in keeping with the work of other groups who have shown that intravenous administration of a bradykinin receptor antagonist does not affect systemic haemodynamic parameters in healthy volunteers [Cockcroft *et al* 1994a].

The discovery and subsequent development of compounds to inhibit ACE arose from research into bradykinin potentiating factor. This protein was found not only to augment the effects of bradykinin on smooth muscle but also to inhibit ACE activity [Ondetti *et al* 1971]. It was later realised that the enzymes responsible for bradykinin degradation (kininase II) and angiotensin II generation (ACE) are the same protein. Therefore, it is not surprising that some of the vascular effects of ACE inhibition are

attributable to the action of bradykinin [Hornig *et al* 1997]. We have shown that in healthy volunteers, ACE inhibition causes an increase in the flow associated vasodilatation of reactive hyperaemia, an effect blocked by HOE-140. In sodium depleted volunteers and patients with hypertension, Gainer *et al* [1998] have shown that the acute hypotensive effects of captopril can be ameliorated by intravenous administration of HOE-140. These studies, however, relate only to the acute effects of ACE inhibition and may not be representative of the vascular effects during chronic ACE inhibition.

The only previous study to assess directly the contribution of bradykinin to basal vascular tone in heart failure showed an apparent, but non-significant, vasoconstriction with intra-arterial HOE-140 in patients treated with enalapril [Davie *et al* 1999]. The apparent disparity between our study and that by Davie *et al* [1999] may reflect the differing pharmacological actions of HOE-140 and B9340. To address this issue, we subsequently assessed the effects of HOE-140 in a limited number of patients, including four subjects who had received B9340. We also found no significant vasoconstrictor effects of HOE-140 in patients with chronic heart failure maintained on ACE inhibitor therapy.

Des-Arg⁹-bradykinin, a selective B₁ receptor agonist, is the product of kininase I action on bradykinin [Lamontagne *et al* 1995]. Theoretically, if ACE (kininase II) is inhibited, bradykinin will be preferentially metabolised to des-Arg⁹-bradykinin. Indeed, there is a suggestion that potentiation of des-Arg⁹-bradykinin may, in part, be responsible for some of the side effects of ACE inhibitor therapy, including angio-oedema [Blais *et al* 1999]. It has been shown in dogs that infusion of des-Arg⁹-

bradykinin causes resistance and conduit vessel dilatation [Su *et al* 2000]. In our study, we have used a relatively non-selective bradykinin receptor antagonist, B9340, which, although retaining a similar activity at the B₂ receptor, has a 100-fold greater affinity for the B₁ receptor than HOE-140 [Stewart *et al* 1997]. Moreover, B₁ receptor expression on vascular endothelium is upregulated in inflammatory conditions [Marceau 1995] and atherosclerosis [Raidoo *et al* 1997], and there is now evidence that inflammation plays an important role in heart failure, with elevated plasma concentrations of circulating cytokines, such as tumor necrosis factor- α and interleukin 1 β [Kelly and Smith 1997]. Thus, the vasoconstrictor effects of B9340 may represent combined inhibition of the vascular actions of des-Arg⁹-bradykinin and bradykinin at the B₁ and B₂ receptors. However, to date, the biological activity and functional significance of des-Arg⁹-bradykinin and B₁ receptor mediated effects have not been assessed in humans.

This study has wide ranging implications for future work on ACE inhibition. If part of the benefit of ACE inhibitor therapy is attributable to increases in endogenous bradykinin and its metabolites, then even greater benefit might be obtained from combined ACE and neutral endopeptidase (NEP) inhibition. Indeed, *in vivo* animal [Raut *et al* 1999] and *ex vivo* human [Kokkonen *et al* 1999] studies have demonstrated that the actions of bradykinin are augmented additionally by combined ACE and NEP inhibition. Moreover, the recent IMPRESS trial [Rouleau *et al* 2000] showed a significant benefit over lisinopril in the combined endpoint of death, hospital stay, or discontinuation of study medication for worsening of heart failure when patients with heart failure were treated for 24 weeks with omapatrilat, a combined ACE and NEP inhibitor. However, combined vaso-peptidase inhibitors like

omapatrilat also augment other peptidic systems, such as the natriuretic peptides, and it remains to be established whether bradykinin provides a greater contribution to the maintenance of basal vascular tone in patients treated with these agents.

5.6 CONCLUSION

In conclusion, B9340 is a potent and specific inhibitor of bradykinin-induced peripheral vasodilatation. Using B9340 at doses sufficient to increase the ED_{300} of bradykinin by 30-fold, we have demonstrated that bradykinin contributes to the maintenance of basal peripheral vascular tone in patients receiving chronic ACE inhibitor therapy for heart failure. Since this work was performed a further study has demonstrated that ACE inhibition augments bradykinin mediated vasodilatation and endogenous tPA release only via the B2 receptor [Cruden *et al* 2005].

CHAPTER 6

BRADYKININ CONTRIBUTES TO THE SYSTEMIC HAEMODYNAMIC EFFECT OF CHRONIC ANGIOTENSIN-CONVERTING ENZYME INHIBITION IN PATIENTS WITH HEART FAILURE

6.1 SUMMARY

Background - Bradykinin is an endogenous vasodilator that may contribute to the systemic effects of ACE inhibitor therapy. Using B9340, a bradykinin receptor antagonist, we determined the contribution of bradykinin to the systemic haemodynamic effects of long-term ACE inhibition in patients with chronic heart failure.

Methods and Results - Fourteen patients with heart failure received enalapril (10 mg twice daily) or losartan (50 mg twice daily) in a randomised double-blind crossover trial. After 6 weeks treatment, patients underwent right heart catheterisation and were randomised to an intravenous infusion of B9340 (2-20 $\mu\text{g/kg/min}$) or saline placebo. After B9340 infusion in patients treated with enalapril, mean arterial pressure (+5.2 mmHg), systemic vascular resistance (+315 dynes·s/cm⁵), pulmonary arterial wedge pressure (-1.4 mm Hg), and mean pulmonary arterial pressure (-1.3 mm Hg) were greater compared with losartan ($p<0.005$, $p<0.07$, $p<0.0001$, and $p<0.05$ respectively) or placebo infusion ($p<0.005$ for all). There was a reduction in cardiac output after B9340 with enalapril compared with placebo ($p<0.001$) but not losartan.

Conclusions - Bradykinin contributes to the systemic haemodynamic effects of long-term ACE inhibition in patients with heart failure. This mechanism may explain the apparent clinical differences between ACE inhibitors and angiotensin receptor blockers in the treatment of heart failure.

6.2 INTRODUCTION

Activation of the renin-angiotensin-aldosterone system plays a key role in the pathogenesis of chronic heart failure, leading to salt and water retention and peripheral vasoconstriction. Inhibitors of ACE reduce peripheral vascular tone and systemic arterial pressure [Powers *et al* 1982] and improve symptoms and survival inpatients with chronic heart failure [The CONSENSUS Trial Study Group 1987]. Although previously attributed to a reduction in angiotensin mediated vasoconstriction, recent data have indicated that the haemodynamic changes associated with ACE inhibitor therapy may be caused, at least in part, by the inhibition of bradykinin metabolism. The bradykinin metabolising properties of ACE (kininase II) were first described in 1967 [Erdös and Yang 1967]. Indeed, ACE has greater affinity for bradykinin than for angiotensin I [Jaspard *et al* 1993] and is the principal enzyme responsible for the rapid turnover of bradykinin (plasma half-life ~15 seconds) [Bonner *et al* 1990]. This rapid metabolism makes accurate measurement of bradykinin difficult, although elevated plasma bradykinin concentrations have been reported in the presence of ACE inhibition [Pellacani *et al* 1994]. Bradykinin is a powerful endothelium-dependent vasodilator and, at a functional level, inhibition of ACE potentiates the action of exogenous bradykinin in the human forearm and coronary circulations [Benjamin *et al* 1989; Kuga *et al* 1997; Witherow *et al* 2002]. There are two principal kinin receptor subtypes in humans, B₁ and B₂ [Stewart *et al* 1997]. The endothelial B₂ receptor is constitutively expressed and mediates the vasodilator and profibrinolytic effects of bradykinin [Cockcroft *et al* 1994a; Brown *et al* 2000]. The vascular B₁ receptor is normally expressed very

weakly but is markedly upregulated in the presence of inflammation, cardiovascular disease states [McLean *et al* 2000] and ACE inhibition [Marin-Castano *et al* 2002], where it also mediates vasodilatation [Drummond and Cocks 1995]. The selective kinin receptor antagonists, HOE-140 (icatibant) and B9340, are synthetic peptide analogues of bradykinin with different inhibitory activities at the specific kinin receptor subtypes. HOE-140 acts solely at the B₂ receptor, whereas B9340 blocks kinin activity mediated via both B₁ and B₂ receptors [Stewart *et al* 1997]. Several investigators have reported that intravenous HOE-140 attenuates the vasodepressor response associated with acute ACE inhibition, both in healthy volunteers and in hypertensive patients [Gainer *et al* 1998; Squire *et al* 2000]. In contrast, B9340, but not HOE-140, induced vasoconstriction in the forearm circulation of patients with heart failure treated with chronic ACE inhibitor therapy [Davie *et al* 1999; Witherow *et al* 2001]. Besides implicating bradykinin in the haemodynamic changes associated with ACE inhibition, these data raise the possibility of a role for the B₁ receptor in patients with chronic heart failure [Witherow *et al* 2001]. The aims of the present study were to demonstrate that the kinin receptor antagonist, B9340, inhibits bradykinin activity in the systemic circulation and to determine whether endogenous bradykinin contributes to the haemodynamic effects of long-term ACE inhibition in patients with chronic symptomatic heart failure.

6.3 METHODS

6.3.1 STUDY SUBJECTS

The protocols were performed with the approval of the Local Research Ethics Committee and in accordance with the Declaration of Helsinki (1989). Written

informed consent was obtained from each subject. Six healthy, male non smokers attended fasted at 8.00 a.m. on two occasions at least 1 week apart. Volunteers avoided alcohol and caffeine for 24 hours before each study and received no medications or vasoactive substance in the preceding 7 days. Fourteen patients with stable NYHA class II–III chronic heart failure and objective evidence of left ventricular impairment (left ventricular ejection fraction $\leq 40\%$, shortening fraction $\leq 20\%$, or left ventricular end diastolic diameter ≤ 5.5 cm) were enrolled into the study. Patients were included only if they had been established on maximally tolerated ACE inhibitor therapy for at least 6 months. Patients were excluded if they had significant valvular heart disease, renal or hepatic failure, or had previous malignant ventricular arrhythmias. After initial screening, patients received enalapril 10 mg [The CONSENSUS Trial Study Group 1987] or losartan 50 mg [Pitt *et al* 2000], both twice daily, in place of their usual ACE inhibitor in a randomised double-blind crossover trial. There was no placebo limb of the trial as it was felt that it would be unethical to have patients with heart failure untreated by some form of angiotensin blockade for such a long period.

After 6 weeks of each treatment, subjects attended fasted at 8.00 a.m. and underwent right heart catheterisation. On the morning of each visit, oral study medication was administered at 7.00 a.m. to achieve peak plasma concentrations of the active metabolites, enalaprilat or E-3174, respectively, during B9340 or placebo infusion (10.00–11.00 a.m.). Diuretics were withheld on the morning of each study for patient comfort.

6.3.2 MEASUREMENTS

Healthy Volunteer Study

Intra-arterial drug administration and FBF measurements were performed using venous occlusion plethysmography as previously described [Witherow *et al* 2001]. After each FBF measurement, heart rate and blood pressure were determined non-invasively in the non-infused arm using a semi-automated, sphygmomanometer (UA-731; A&D Engineering, Milpitas, Calif).

Patient Study

A 9-French venous sheath was inserted aseptically under local anaesthesia via the right femoral vein. Under fluoroscopic screening, a continuous CO thermodilution Swan-Ganz catheter (Edwards Lifesciences, Irvine, Calif) was positioned in the pulmonary arterial tree. Pulmonary arterial pressure, pulmonary arterial wedge pressure, and central venous pressure were recorded using a Hewlett Packard monitor (U78339A; Hewlett Packard, Andover, Mass). Continuous cardiac output was recorded using a Vigilance monitor (Edwards Lifesciences, Irvine, Calif). Heart rate and blood pressure were measured non-invasively using a semi-automated sphygmomanometer (U78339A; Hewlett Packard, Andover, Mass).

6.3.3 DRUGS

B9340 (molecular weight 1318.6) is a synthetic peptide antagonist of bradykinin with potent inhibitory activity at both the B₁ and B₂ receptors (pIC₅₀ *in vitro* of 8.1 and 9.8, respectively) [Stewart *et al* 1997]. The doses of B9340 and bradykinin were chosen after the results of dose ranging studies performed in the forearm circulation

of healthy volunteers [Witherow *et al* 2003]. Pharmaceutical grade B9340 and bradykinin were supplied by Clinalfa AG (Laufelfingen, Switzerland) and dissolved in saline on the day of study.

6.3.4 PROTOCOL DESIGN:

Healthy Volunteer Study

After 30 minutes equilibration with saline infusion, bradykinin was infused via the brachial artery of the non-dominant arm at 300 pmol/min for 120 minutes. B9340 or saline placebo was infused intravenously in the contralateral arm at 2, 6, and 20 µg/kg/min for 12 minutes at each dose with 12 minutes separating each dose. Forearm blood flow was measured at 6 minute intervals throughout each study.

Patient Study

After a 30 minute infusion of 50 mL saline, patients received an intravenous infusion of B9340 at 2, 6, and 20 µg/kg/min for 15 minutes at each dose or saline placebo (75 mL) in a randomised, double-blind manner. The randomisation was weighted such that 10 patients on enalapril received B9340, 10 patients on losartan received B9340, and six patients on either enalapril or losartan received placebo. Haemodynamic measurements were recorded at -40, -30, -10, 0 (baseline), 7, 15, 22, 30, 37, 45, 60, 75, 90, and 105 minutes during each study. Venous blood was collected at 0 (baseline), 45, and 105 minutes for determination of plasma ACE activity and plasma angiotensin II concentrations.

6.3.5 LABORATORY ANALYSIS

Blood samples were collected on ice, centrifuged immediately, and the resulting supernatant stored at -70°C until assayed. Plasma ACE activity was determined using colorimetric spectrophotometry (SIGMA Diagnostics, St Louis, Mo) (Holmquist *et al* 1979). After extraction using Bond Elut columns (Varian; Harbor City, Calif), plasma angiotensin II (Diasorin, Stillwater, Minn) concentrations were determined by radioimmunoassay [Morton and Webb 1985].

6.3.6 DATA ANALYSIS AND STATISTICS

Data are expressed as mean \pm standard error of the mean. Mean arterial pressure was defined as diastolic pressure plus a third of the pulse pressure. Forearm blood flows were calculated from plethysmographic data as described previously [Witherow *et al* 2001]. In patient studies, haemodynamic parameters were assessed over time as absolute change from baseline (0 minutes) using 1-way ANOVA with repeated measures. Comparisons between treatment groups were made using 2-way ANOVA for which the within subject variables were drug and time. Plasma neurohormone concentrations were compared at baseline using an unpaired *t*-test. Statistical significance was taken at the 5% level.

6.4 RESULTS

Healthy Volunteer Study

Bradykinin caused a sustained increase in FBF during placebo coinfusion ($p < 0.0001$; Figure 6.1). Systemic infusion of B9340 inhibited bradykinin mediated vasodilatation in a dose-dependent manner ($p < 0.0001$; Figure 6.1).

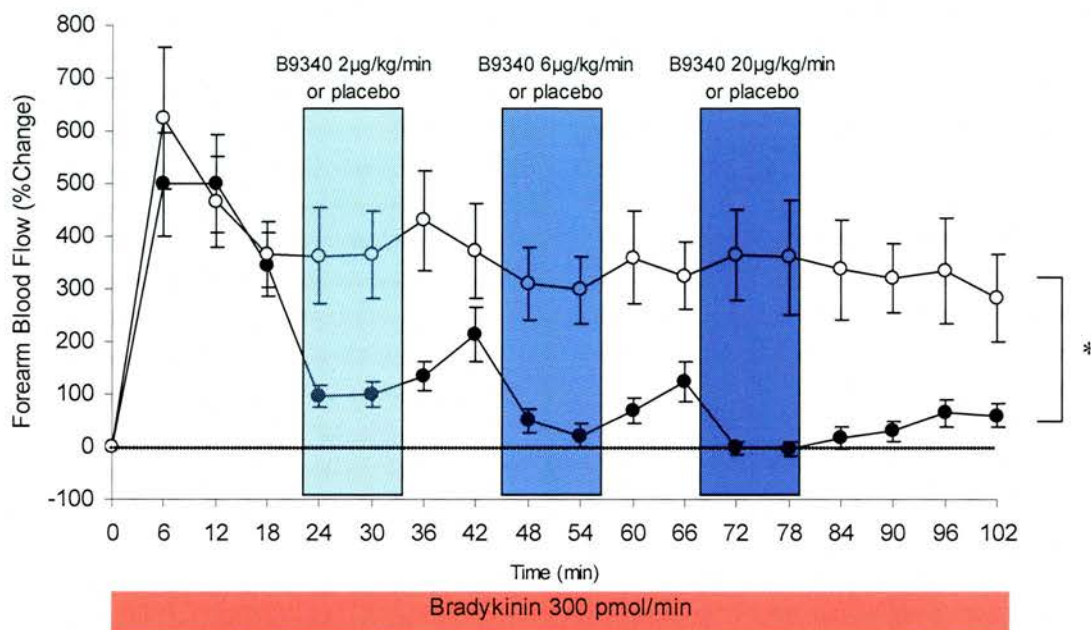


Figure 6.1 Effect of systemic B9340 (2 to 20 µg/kg per minute; closed circles) and placebo infusion (open circles) on bradykinin mediated forearm vasodilatation in healthy volunteers, *p<0.001.

Patient Study

Fourteen patients participated in the haemodynamic study. After the first visit, one patient withdrew because of worsening heart failure unrelated to treatment and was replaced. There were no significant differences in patient characteristics or baseline haemodynamic parameters between treatment groups (Table 6.1).

	Enalapril + B9340 Infusion	Losartan + B9340 Infusion	Enalapril/Losartan* + Placebo Infusion
	(n_10)	(n_10)	(n_6)
Age in years (range)	62 (45–75)	63 (45–78)	66 (60–73)
Gender (male/female)	8/2	9/1	5/1
Diagnosis (IHD/DCM)	9/1	8/2	5/1
NYHA class (II/III)	8/2	6/4	3/3
Ejection fraction (%)	28 (3)	28 (3)	31 (2)
Body mass index	26 (1)	26 (1)	25 (2)
Concomitant medications			
Aspirin	8	8	6
Diuretic	9	8	5
Beta-blocker	7	6	3
Statin	7	6	5
Nitrate	3	3	3
Digoxin	2	1	3
Calcium antagonist	1	2	1
Spironolactone	0	1	1
Baseline hemodynamics			
Heart rate (beats/min)	62 (3)	62 (4)	61 (3)
MAP (mm Hg)	81 (4)	83 (4)	83 (3)
SVR (dynes.s/cm ⁵)	1228 (116)	1194 (110)	1443 (80)
Cardiac output (L/min)	5.4 (0.4)	5.6 (0.5)	4.7 (0.2)
CVP (mm Hg)	1 (1)	1 (1)	1 (1)
MPAP (mm Hg)	15 (2)	18 (2)	15 (2)
PAWP (mm Hg)	5 (1)	9 (2)	7 (1)

Plasma Neurohormones

Plasma ACE activity and plasma angiotensin II concentrations at baseline on the morning of haemodynamic studies were significantly lower in patients treated with enalapril compared with losartan respectively (Figure 6.2). There were no significant changes in plasma ACE activity or angiotensin II concentrations during or after B9340 or placebo infusion.

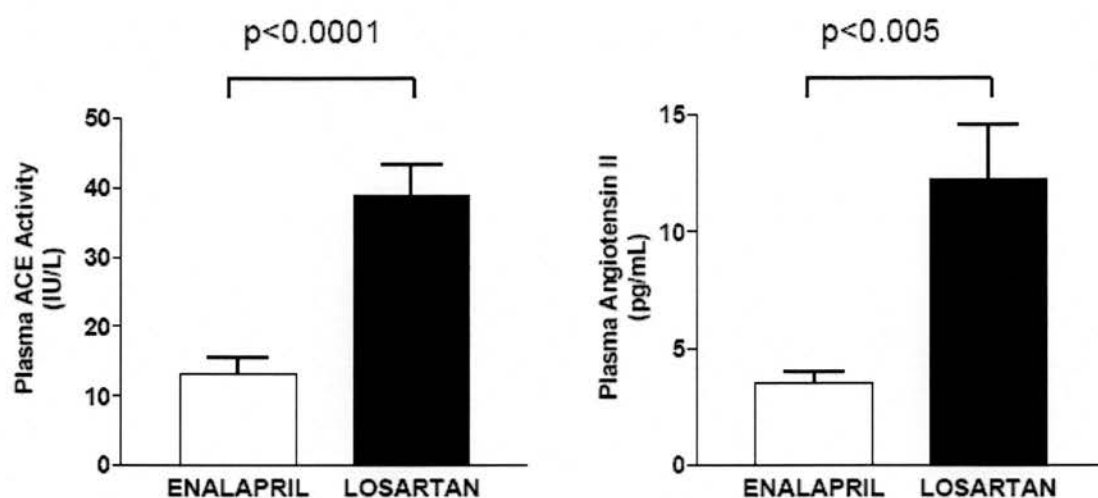


Figure 6.2 Baseline plasma ACE activity (n=14) and angiotensin II (n=13) concentrations in patients with heart failure after 6 weeks of therapy with enalapril (open bar) or losartan (solid bar).

Mean Arterial Pressure and Heart Rate

There were no significant changes in heart rate or mean arterial pressure during placebo infusion or after losartan therapy. Mean arterial pressure increased after administration of B9340 in patients treated with enalapril ($p<0.005$; Figure 6.3), although there was no significant change in heart rate. This pressor effect was greater in patients treated with enalapril than those given losartan therapy or placebo infusion ($p<0.005$ and $p<0.0001$, respectively; Figure 6.3).

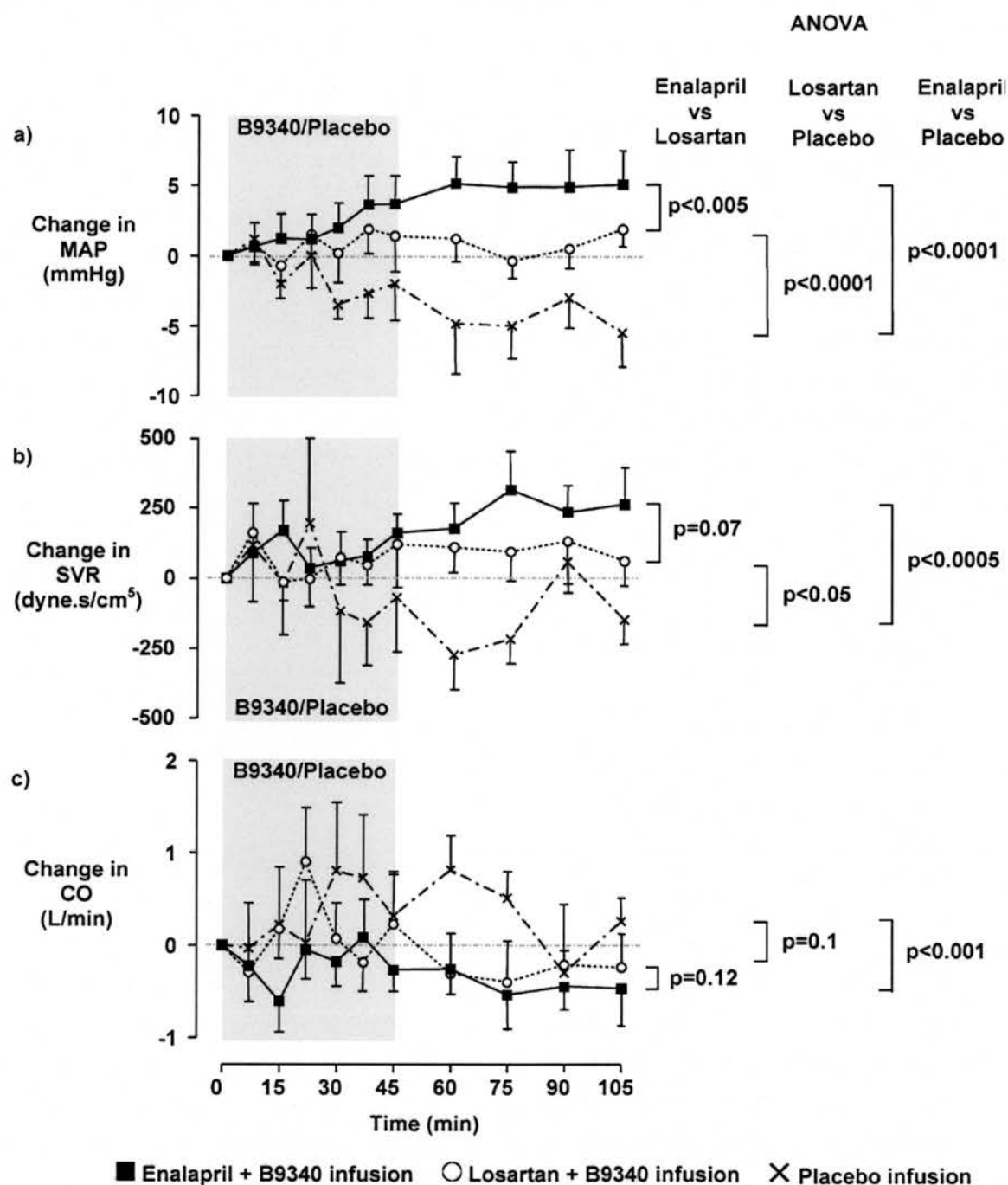


Figure 6.3 Changes in (a) mean arterial pressure (MAP), (b) systemic vascular resistance (SVR), and (c) cardiac output (CO) after intravenous infusion of saline placebo (crosses) or B9340 (2 to 20 μ g/kg per minute) in patients with chronic heart failure treated with enalapril (black squares) or losartan (white circles).

There were no significant changes in cardiac output or systemic vascular resistance during placebo infusion or after losartan therapy. In patients treated with enalapril receiving B9340, there was a trend toward an increase in systemic vascular resistance ($p<0.08$) but no change in CO.

Central Pressures

Central venous pressure, pulmonary arterial wedge pressure, and mean pulmonary arterial pressure all decreased significantly over time during placebo infusion ($p<0.05$ for all; Figure 6.3). Compared with losartan therapy or placebo infusion, B9340 attenuated the decrease in pulmonary arterial wedge pressure ($p<0.0001$ for both) and mean pulmonary arterial pressure ($p<0.05$ and $p<0.005$, respectively) in patients treated with enalapril (Figure 6.3). There were no significant differences in the reductions of central venous pressure between treatment groups (Figure 6.3).

Adverse Events

There were no major drug related adverse events reported during healthy volunteer or patient studies. Three healthy volunteers and one patient reported mild self-limiting diarrhoea after B9340 infusion.

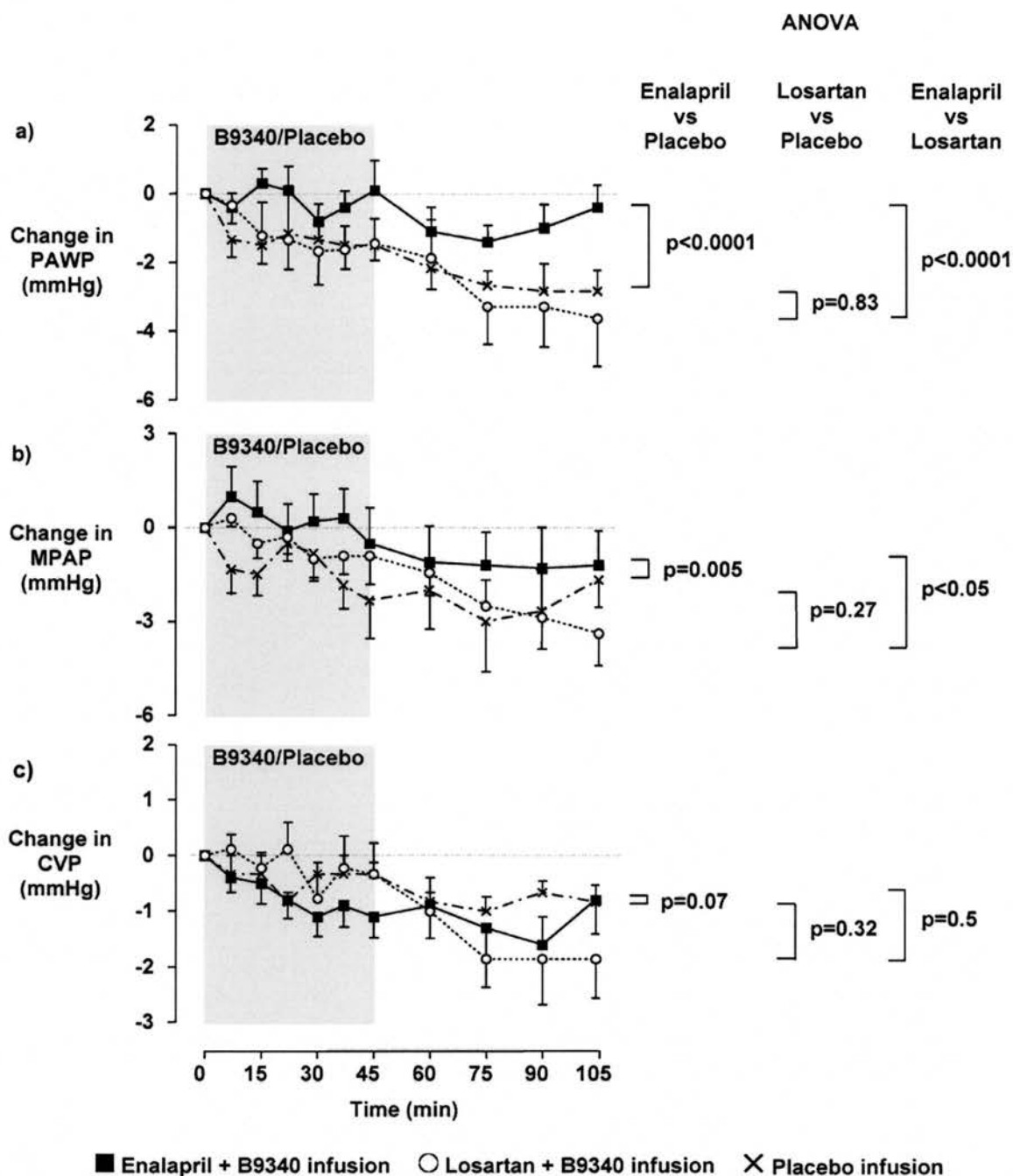


Figure 6.4 Changes in (a) pulmonary arterial wedge pressure (PAWP), (b) mean pulmonary arterial pressure (MPAP), and (c) central venous pressure (CVP) after intravenous infusion of saline placebo (crosses) or B9340 (2 to 20 $\mu\text{g/kg}$ per minute) in patients with chronic heart failure treated with enalapril (closed squares) or losartan (open circles).

6.5 DISCUSSION

We have demonstrated that intravenous B9340, a selective peptidic antagonist of bradykinin receptors, causes systemic inhibition of bradykinin mediated vasodilatation in a dose-dependent manner. This bradykinin receptor antagonist has a significant pressor effect in patients with chronic heart failure maintained on long-term ACE inhibitor but not angiotensin receptor blocker therapy. These findings provide convincing evidence that bradykinin contributes to the hypotensive effects of chronic ACE inhibitor therapy in patients with heart failure. We have previously shown that B9340 causes vasoconstriction in the forearm circulation in patients with heart failure treated with long-term ACE inhibitor therapy [Witherow *et al* 2001]. The results of the present study confirm that these effects are important in the systemic circulation. This is consistent with published data demonstrating that the bradykinin receptor antagonist, HOE-140, attenuates the acute vasodepressor response to ACE inhibition. Gainer *et al* [1998] demonstrated that in salt-deplete healthy volunteers and hypertensive subjects, a systemic infusion of HOE-140 attenuated the hypotensive response to a single dose of captopril. The reduction in blood pressure observed after coadministration of HOE-140 and captopril was similar to that seen with the angiotensin receptor blocker, losartan [Gainer *et al* 1998]. As the authors point out, previous studies have demonstrated that the peak hypotensive responses to captopril and losartan occur 1 and 4 hours, respectively, after drug administration [Gainer *et al* 1998]. It has been suggested that these differences could account for the observed differences in the blood pressure response. To overcome this potential methodological difficulty, we chose to compare

the ACE inhibitor, enalapril, at a dose previously shown to reduce mortality in patients with heart failure [The CONSENSUS Trial Study Group 1987] with the angiotensin receptor antagonist, losartan, administered at the maximum daily dose. Both these agents have long-acting active metabolites, enalaprilat and E-3174, respectively, achieving peak plasma concentrations 3 to 4 hours after ingestion that coincided with systemic intravenous administration of B9340 or placebo [Abrams *et al* 1984; Ohtawa *et al* 1993]. It should also be recognised that we have assessed the effects of kinin receptor blockade in patients maintained on long-term ACE inhibitor therapy rather than the acute effects of a single oral dose. Moreover, patients with heart failure maintained on chronic ACE inhibitor therapy may upregulate vascular B₁ kinin receptor expression [Witherow *et al* 2001] and we therefore used a combined B₁ and B₂ receptor antagonist to characterise more precisely the role of bradykinin.

It has been argued [Schiffrin 2002] that angiotensin receptor blockade might potentially be more effective at blocking the detrimental effects of angiotensin II than ACE inhibition because in a substantial proportion of patients treated with chronic ACE inhibition, plasma angiotensin II concentrations increase over time returning toward pretreatment values [MacFadyen *et al* 1999]. This “ACE escape” is thought to be caused by angiotensin II formation by non ACE dependent pathways and may be associated with a poorer prognosis [Petrie *et al* 2001]. However, clinical evidence that angiotensin receptor blockers have superior efficacy to ACE inhibitor therapy has proved elusive. In contrast, recent trials have confirmed that the therapeutic benefits of ACE inhibitors and angiotensin receptor blockers are additive:

combination therapy improving symptoms and cardiovascular mortality in patients with heart failure when compared with ACE inhibition alone [Cohn and Tognoni 2001; McMurray *et al* 2003]. Our findings may help to explain the additive benefits of combined ACE inhibition and angiotensin receptor blockade. Large scale clinical trials have demonstrated that ACE inhibitors not only improve survival but reduce the incidence of myocardial ischaemia [Flather *et al* 2000]. This may be explained, in part, by the observation that ACE inhibition improves the fibrinolytic balance in patients with heart failure and ischaemic heart disease [Wright *et al* 1994]. Bradykinin is not only a potent vasodilator but is intimately involved with the coagulation and fibrinolytic cascades. Indeed, it is a powerful mediator of endogenous fibrinolysis through the release of endothelium-derived t-PA [Brown *et al* 2000; Witherow *et al* 2002; Pretorius *et al* 2003]. In patients with heart failure, chronic ACE inhibition markedly augments local bradykinin mediated release of t-PA [Witherow *et al* 2002]. Thus, potentiation of the other vascular actions of bradykinin may also contribute to the clinical benefits of ACE inhibitor therapy.

In patients treated with losartan, there was a small but significant increase in mean arterial pressure and systemic vascular resistance after B9340 infusion compared with placebo. This may have reflected the significant protein load associated with B9340 infusion and a protein based placebo, such as albumin, may have been a more appropriate control. There are data indicating that bradykinin may contribute to the vascular effects of angiotensin receptor blockers. In transgenic mice overexpressing the AT₂ receptor, angiotensin II causes vasodilatation that is attenuated by HOE-140 [Tsutsumi *et al* 1999] and in human, HOE-140 inhibits the improvement in flow

mediated vasodilatation associated with the angiotensin receptor blocker, candesartan [Hornig *et al* 2003]. Finally, the findings are consistent with the possibility that bradykinin also contributes to the maintenance of blood pressure and vascular tone in patients with heart failure independently of ACE.

In summary, we have shown that in patients with chronic heart failure, systemic infusion of the combined kinin receptor antagonist, B9340, attenuates the vasodepressor effects associated with long-term enalapril therapy when compared with treatment with the angiotensin receptor blocker, losartan.

6.6 CONCLUSION

We conclude that in patients with chronic symptomatic heart failure, bradykinin contributes to the systemic haemodynamic effects associated with long-term ACE inhibitor therapy.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 BRADYKININ MEDIATED STIMULATION OF THE ENDOTHELIUM

Stimulation of the forearm vascular bed with bradykinin is associated with a selective and reproducible vasodilatation and release of t-PA at intrabrachial doses of 30 nmol/min or higher. This endothelial cell stimulation can be inhibited by B9340 a bradykinin receptor antagonist with high affinity for the B₂ receptor. B9340 failed to inhibit substance P mediated dilation and t-PA release thus confirming that bradykinin stimulates the endothelium via a specific receptor rather than a generalised mechanism. Furthermore bradykinin mediated forearm vasodilatation and endothelial t-PA release was enhanced by the presence but not the absence of ACE inhibition in patients with heart failure.

7.1.1 BRADYKININ MEDIATED TISSUE PLASMINOGEN ACTIVATOR RELEASE

The ability of intra-arterial bradykinin to release t-PA from the endothelium can be reproduced by other stimulants. Substance P can also produce rapid mobilisation of t-PA from endothelial storage pools and is slightly more potent than bradykinin, and desmopressin can also stimulate endothelial t-PA release [Newby *et al* 2000]. However not all endothelial stimulants are the same, as no discernable rise in t-PA was seen after acetylcholine infusion despite producing vasodilatation [Brown *et al* 2000]. The potent vasoconstrictor angiotensin II did not produce any acute change in plasma t-PA or PAI-1 levels [Labinjoh *et al* 2000], despite drug levels that produced both local and systemic vasoconstrictor effects. Also increasing blood flow per se does not release t-PA from the endothelium, as the endothelium independent

vasodilator sodium nitroprusside did not produce any rise in t-PA or PAI-1 levels despite producing marked vasodilatation [Newby *et al* 1997]. Thus it would seem that intravascular t-PA release is specific to a few agonists acting on specific receptors on the endothelium. It is of note however that kinin mediated endothelial t-PA release can be increased by inflammation without a significant increase in blood flow [Chia *et al*, 2003a].

The ability of the endothelium to produce enormous amounts of t-PA has been shown in Chapter 4. At an infusion rate of 300 pmol/min of bradykinin, the maximal local forearm concentrations of active t-PA (99 IU/ml) approached those observed during systemic thrombolysis during acute myocardial infarction (100 to 1,000 IU/ml) (Koster). Moreover, it also underscores the ability of the endothelium to release t-PA quickly, up to 4.5 µg or 16,000 IU/min from the infused forearm at 300 pmol/min of bradykinin. This ability to release t-PA seems to be sustainable at least for 2 hours [Newby *et al* 1997].

7.1.2 PLASMINOGEN ACTIVATOR INHIBITOR

It is also of note that despite the potent stimulation of t-PA release by substance P and bradykinin there was no discernible acute release of PAI-1 from the endothelium. Plasminogen activator inhibitor 1 is also produced in the endothelium but does not seem to have the same ability to be rapidly mobilised despite its role in deactivating t-PA by binding it in a t-PA/PAI-1 complex. Similarly in the human coronary circulation, bradykinin stimulates endothelial release of t-PA without affecting plasma concentrations of PAI-1 [Minai *et al* 2001]. This seeming disparity

between t-PA and PAI-1 would be advantageous when endothelium becomes damaged, as the damaged area is procoagulant and the subsequent limitation of blood clot propagation requires a rapid response from the endothelium to avoid ischaemia in the distal bed.

7.2 ANGIOTENSIN-CONVERTING ENZYME INHIBITION AND TISSUE PLASMINOGEN ACTIVATOR

The clinical and therapeutic benefits of inhibition of angiotensin-converting enzyme has lead to the widespread use of ACE inhibitor therapy to treat a variety of cardiovascular disorders including hypertension, myocardial infarction, vascular disease and left ventricular dysfunction. Treatment with ACE inhibition tends to produce vasodilatation and subsequent lowering of blood pressure in humans and some of the haemodynamic effects may be due in part to enhanced bradykinin activity in the vasculature. In addition to the haemodynamic effects, there is evidence that bradykinin contributes to the increase in constitutive t-PA release associated with ACE inhibitor therapy by a B₂ receptor mechanism. In the forearm circulation of healthy smokers, intra-arterial enalaprilat increased net basal t-PA release and this effect was blocked by pre-treatment with HOE-140 [Pretorius *et al* 2003].

In this thesis, we confirmed earlier findings in healthy volunteers that bradykinin stimulated t-PA release is augmented by treatment with ACE inhibition, [Labinjoh *et al* 2001]. However, it is of note that the amount of t-PA released by patients with heart failure treated with chronic ACE inhibitor therapy in this study, was much

greater than that of healthy volunteers treated with ACE inhibition in the study by Labinjoh *et al* [2001]. At a bradykinin infusion rate of 300 pmol/min, the t-PA antigen concentration in infused arm was 11.7 ± 1.7 ng/ml in healthy volunteers and 33.9 ± 1.7 ng/ml in patients despite similar baseline levels of t-PA antigen. The more accurate measure of t-PA production, the net t-PA release across the forearm vascular bed shows that the ability of the patients with heart failure to release t-PA is also greater (259.8 ± 22 ng/100ml/min) than healthy volunteers (90 ± 22.9 ng/100ml/min) when stimulated by 300 pmol/min of intrabrachial bradykinin. This difference could be accounted for by the difference in age and size of the patients compared to the volunteers, however if t-PA activity is compared between the two different groups it can be shown that not only do the heart failure patients treated with ACE inhibition have much higher basal levels of t-PA activity but also a far greater ability to rapidly produce active t-PA. Interestingly when ACE inhibition is withdrawn, the ability of the patients with heart failure to release t-PA is reduced when compared to the healthy volunteers, demonstrating the endothelial dysfunction that occurs in patients with heart failure. Thus it would seem that the benefits of ACE inhibition on endothelial t-PA release are greater in disease than in health. This may be part of the explanation why greater benefits of ACE inhibitor therapy are seen in more severe disease such as heart failure with severe left ventricular impairment [The CONSENSUS Trial Study Group 1987] compared to high risk vascular disease without left ventricular impairment [The HOPE Study Investigators 2000].

7.3 ANGIOTENSIN-CONVERTING ENZYME INHIBITION AND PLASMINOGEN ACTIVATOR INHIBITOR

In Chapter 4 it was also seen that basal PAI-1 levels were greater in the absence than in the presence of ACE inhibitor therapy. This confirms previous data that ACE inhibition lowers levels of PAI-1 in patients with heart failure. [Vaughan *et al* 1995b; Goodfield *et al* 1999]. As the basal level of PAI-1 is lower there will be less PAI-1 to bind and inactivate t-PA showing that ACE inhibition not only increases the amount of t-PA released in response to bradykinin stimulation but also reduces the amount of inactivation of t-PA.

7.4 BRADYKININ MEDIATED VASODILATATION

Intravenous administration of bradykinin results in arteriolar vasodilatation and a rapidly reversible fall in blood pressure and peripheral vascular resistance [Bonner *et al* 1990], and intra-arterial administration produces local vasodilatation [Labinjoh *et al* 2001]. In this thesis, it has been demonstrated that endogenous bradykinin does not appear to contribute to the maintenance of peripheral vascular tone in healthy volunteers or in patients with heart failure which confirms the work of others who have shown that local, intra-arterial infusion of HOE-140 has no effect on resting blood flow [Hornig *et al* 1997; Davie *et al* 1999], and that intravenous administration of HOE-140 has no effect on systemic blood pressure [Gainer *et al* 1998]. We have also shown that in the human peripheral circulation, exogenous bradykinin causes a dose-dependent increase in blood flow through an endothelium and bradykinin B₂

receptor dependent mechanism. As has been demonstrated by previous authors, intrabrachial bradykinin administration appears to be well tolerated, with only minor discomfort and mild oedema at high doses. (Cockcroft *et al* 1994a; Brown *et al* 2000; Labinjoh *et al* 2001).

7.5 HAEMODYNAMIC EFFECTS OF ACE INHIBITION AND ANGIOTENSIN RECEPTOR BLOCKADE

As has been mentioned previously, angiotensin-converting enzyme's dual ability of activating angiotensin I and inactivating bradykinin has led to the extensive investigation of the enzyme and subsequent use of converting enzyme inhibitors and angiotensin II receptor blockers in the treatment of cardiovascular disease. It has been previously demonstrated that some of the acute effects of ACE inhibition on blood pressure are due to bradykinin. Gainer *et al* [1998] reported that a systemic infusion of HOE-140 attenuates the systemic response to a single oral dose of captopril. The reduction in blood pressure observed following coadministration of HOE-140 and captopril was similar to that seen with the angiotensin receptor blocker losartan. In the Chapters 5 and 6, it has been shown that infusion of intra-arterial and intravenous B9340 produces vasoconstriction in patients with heart failure treated with chronic ACE inhibition but not in the absence of ACE inhibition. This strongly suggests that ACE inhibition mediates some of its vasodilator effects via bradykinin. We have also shown for the first time that ACE inhibition augments bradykinin-induced vasodilatation in patients with heart failure, although it has been previously demonstrated in healthy volunteers (Benjamin *et al* 1989; Labinjoh *et al* 2001).

In Chapter 5, vasoconstriction in the ACE inhibitor treated group of heart failure patients was mediated by B9340 and not HOE-140. From this it can be assumed that some of the vasodilatation of chronic ACE inhibition must be B₁ receptor mediated. Stimulation of the B₁ receptor with intra-arterial des-Arg-bradykinin in coronary arteries in dogs has been shown to produce vasodilatation [Su *et al* 2000], although the mechanism of the vasodilatation needs further elucidation. It has also been reported that having certain polymorphisms of the B₁ receptor gene have been shown to increase cardiovascular risk in hypertensive patients [Dhamrait *et al* 2003]. B₁ receptor upregulation has been shown *in vitro* to be rapidly induced by inflammation [Sardi *et al* 1998; Sardi *et al* 1999] in the form of bacterial lipopolysaccharide and interleukin 1 β . Patients with heart failure also have increased levels of inflammatory markers so this may explain why B9340 and not HOE-140 produced a local vasoconstriction in this study.

7.6 ANGIOTENSIN RECEPTOR BLOCKADE AND BRADYKININ

In Chapter 6 we confirmed that our findings in the peripheral circulation also held true in the systemic circulation. We initially demonstrated the safety of intravenous B9340 in healthy volunteers and showed that it inhibited local exogenous bradykinin. In patients with heart failure we were able to directly compare the systemic bradykinin mediated effects of enalapril with losartan. It has previously been postulated that by blocking the angiotensin II type-1 (AT₁) receptor there would be a greater bioavailability of angiotensin II to stimulate the angiotensin type-II (AT₂)

receptor leading to vasodilatation and that some of this vasodilatation is mediated by bradykinin [Gohlke *et al* 1998]. Previous authors have also shown that HOE-140 can inhibit the improvement seen in flow-mediated dilatation in healthy volunteers treated with candesartan [Hornig *et al* 2003] and more recently elevated bradykinin plasma levels have been found in patients with hypertension treated with losartan [Campbell *et al* 2005].

In Chapter 6 our study showed there was a difference between the effects of placebo and B9340 on systemic haemodynamics in the losartan treated patients. In the losartan treated group of patients there was a non-significant rise in SVR during B9340 infusion, but when this change in SVR was compared to placebo infusion it was different as placebo infusion tended to produce a reduction in SVR. This difference may be attributed to the significant protein load of the B9340 infusion, however it may be that losartan may have systemic vasomotor effects mediated through bradykinin in this group of patients.

7.7 FUTURE DIRECTIONS

Further investigation of the role of bradykinin in the regulation of vascular tone and endogenous fibrinolysis should involve direct investigation of the bradykinin B₁ receptor, in both health and disease states. Further work should also concentrate on elucidating the role of the other enzymes that degrade bradykinin namely NEP and carboxypeptidase U also known as thrombin activatable fibrinolysis inhibitor (TAFI). The biological activity of bradykinin degradation products, especially

bradykinin 1-5, in the human vasculature should be addressed and finally, future work should address whether the effects of bradykinin on endogenous fibrinolysis also hold true in the human coronary circulation.

7.7.1 THE BRADYKININ B₁ RECEPTOR

Stimulation of the bradykinin B₁ receptor produces vasodilatation in both conductance and resistance vessels in dogs [Su *et al* 2000], and in the rodent kidney, heart, and vasculature, B₁ receptor stimulation plays a role in the hypotensive effect of ACE inhibitor therapy [Marin Castaño *et al* 2002]. Angiotensin-converting enzyme inhibitors themselves may also be able to induce expression of the B₁ receptor by a mechanism dependent on direct stimulation of the B₁ receptor itself [Marin Castaño *et al* 2002]. Induction of the B₁ receptor can be rapidly produced by cytokines, and also by ischaemia, atherosclerosis (Marceau *et al* 1998, McLean *et al* 2000) and acute myocardial infarction [Tschope *et al* 2000]. During these conditions, vasodilatation of the affected vascular bed would prove advantageous to help improve ischaemia. The induction and activation of the B₁ receptor, as a mediator of vasodilatation may be one of the protective mechanisms initiated during such conditions and this mechanism may be enhanced by ACE inhibition. Further study of the B₁ receptor in humans is required to ascertain whether B₁ kinin receptor stimulation results in vasodilatation and t-PA release and B₁ receptor antagonism causes vasoconstriction in patients with heart failure but not healthy volunteers.

7.7.2 VASOPEPTIDASE INHIBITION.

As we have shown, bradykinin has beneficial effects on the vasculature including vasodilatation and enhanced fibrinolysis. These benefits are enhanced by blockade of angiotensin-converting enzyme. Increasing the bioavailability of bradykinin further may be achieved by also blocking the enzyme NEP 24.11 also called neprilysin. Neutral endopeptidase leaves bradykinin in a similar manner to ACE, releasing the C-terminal dipeptide of bradykinin. Vasopeptidase inhibition refers to single molecules that block both enzymes simultaneously e.g. omapatrilat. The OVERTURE study assigned 5,770 patients with NYHA class II-IV heart failure to treatment with either enalapril (10 mg twice daily) or omapatrilat (40 mg once daily) for a mean of 14.6 months [Packer *et al* 2002)]. The primary endpoint of combined risk of death or hospitalisation for heart failure requiring intravenous treatment was not different for the two treatment groups, although analysis of secondary outcomes showed the omapatrilat group had a 9% lower risk of cardiovascular death or hospitalisation ($p<0.024$) and a 6% lower risk of death ($p<0.34$). Post-hoc analysis showed an 11% lower risk for hospitalisation for heart failure in patients treated with omapatrilat ($p<0.012$). Although this study did not show superiority in the primary endpoint, there was reduction in cardiovascular mortality that may be due to the beneficial effects of vasopeptidase inhibition on endogenous fibrinolysis. Further studies of vasopeptidase inhibition are required to ascertain whether B_1 and B_2 kinin receptor agonism and antagonism is potentiated by vasopeptidase inhibition.

7.7.3 THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR

Thrombin activatable fibrinolysis inhibitor is a latent plasma procarboxypeptidase also known as carboxypeptidase R, carboxypeptidase U, and procarboxypeptidase B [Campbell and Okada 1989; Hendriks *et al* 1989; Eaton *et al* 1991]. Thrombin activatable fibrinolysis inhibitor is now recognised as the second physiological substrate activated by the thrombin/thrombomodulin complex, the other being protein C. It is important for dampening fibrinolysis by removal of plasmin-exposed lysines on partially digested fibrin clots thereby restricting t-PA binding and further activation of plasminogen [Bajzar *et al* 1995; Redlitz *et al* 1995]. Thus, activated protein C (aPC) and TAFIa may play complementary roles in haemostasis, with aPC dampening the clotting cascade and preventing excessive thrombin generation, while TAFIa serves to protect the clot already formed at the site of injury. As well as restricting t-PA activity, TAFI can also inactivate kinins by removing the C-terminal arginine. Cleavage studies have shown that TAFIa is in fact 10-fold more efficient than carboxypeptidase N (kininase I) in hydrolysing bradykinin *in vitro* to des-Arg-bradykinin [Myles *et al* 2003]. By inactivating bradykinin it further reduces the t-PA activity by reducing t-PA release from the endothelium. Several TAFI inhibitors are currently under development and may lead to potentially novel and important therapies in the treatment and prevention of coronary artery disease.[Chang *et al* 2000; Suzuki K *et al* 2004]. Further study is required to assess the affects of TAFI inhibition on bradykinin mediated vasodilatation and t-PA release.

7.7.4 BRADYKININ 1-5

Inactivation of bradykinin can result in the creation of several fragments which were originally thought to be inactive. Angiotensin-converting enzyme is able to cleave bradykinin at two sites, resulting in bradykinin 1-5 (RPPGF or thrombostatin) or bradykinin 1-7. Bradykinin 1-5 has recently been shown to be the stable metabolite in plasma [Murphey *et al* 2000] and can be measured by liquid chromatography tandem mass spectrometry with electrospray ionisation [Murphey *et al* 2001]. Thrombostatin is an inhibitor of α -thrombin induced platelet activation and an inhibitor of both γ - and α -thrombin induced platelet aggregation and secretion without inhibiting adenosine diphosphate or collagen induced platelet activation [Hassan *et al* 1999]. Thrombostatin does not inhibit thrombin binding to platelets and is as effective as aspirin in preventing electrolytic injury-induced left circumflex coronary artery thrombosis in dogs with combined thrombostatin and aspirin therapy resulting in a greater antithrombotic effect than aspirin alone [Hassan *et al* 1999]. In an *ex vivo* model of balloon angioplasty injury to the vessel wall, thrombostatin was as effective as aspirin in preventing platelet adherence to the vessel wall and fibrin formation [Prieto *et al* 2002]. Although thrombostatin has clear antithrombotic effects in animals, further studies are required to ascertain whether thrombostatin has a role in endogenous fibrinolysis in the human vasculature.

7.7.5 CORONARY CIRCULATION

Bradykinin is known to regulate resting and flow-mediated epicardial tone. Infusion of the B₂ receptor antagonist icatibant into left main coronary arteries of patients with significant coronary stenosis resulted in reduction in arterial luminal area, increased

coronary vascular resistance, and decreased coronary blood flow (Groves *et al.* 1995). Patients with significant coronary artery disease demonstrate reduced coronary artery vasodilation in response to BK (Kuga *et al.* 1995). In patients with mild atherosclerosis or its risk factors, BK-induced coronary artery vasomotion was near normal; however, BK-induced vasodilation was depressed following epicardial stress induced with pacing (Prasad *et al.* 2000). A study comparing expression of kinin receptors on normal and atheromatous blood vessels most strikingly revealed increased B₁ receptor expression on multiple cells within the atheromatous vessels (Raidoo *et al.* 1997). The cells showing increased B₁ receptor immunostaining included endothelial cells, foamy macrophages, inflammatory cells, proliferating fibroblasts, and smooth muscle cells.

Different vascular beds are able to release different amounts of t-PA, such that the upper limbs release four times the amount of the lower limbs [Keber 1988]. The ability of the coronary circulation to acutely release t-PA has been demonstrated by stimulation with intracoronary infusion of substance P [Newby *et al.* 2001a], bradykinin and acetylcholine [Minai *et al.* 2001]. It has also been shown that ACE inhibition but not angiotensin receptor blockade augments the release of t-PA from the coronary circulation [Matsumoto *et al.* 2003]. These findings confirm our own in the peripheral circulation but have not been studied in patients with atheromatous coronary disease or heart failure.

7.8 CLINICAL RELEVANCE

It has been previously demonstrated that there is a clear link between impaired endothelial release of t-PA and risk factors for the progression of atherosclerosis. In man, the capacity of the coronary circulation *in vivo* to release t-PA in response to acute stimulation with substance P, is inversely associated with increasing atheromatous plaque burden and smoking habit [Newby *et al* 1999b]. Moreover, both bradykinin and substance P mediated endothelial t-PA release are impaired in the forearm circulation of cigarette smokers. [Newby *et al* 1999b; Pretorius *et al* 2002]. Given that the vasomotor effects of bradykinin in the human coronary circulation are attenuated at sites of atheromatous plaque [Kuga *et al* 1995], it is likely that bradykinin mediated endothelial t-PA release may also be impaired.

In the early studies of ACE inhibitor therapy in heart failure it was noted that the reduction in mortality in the treatment arms of the studies was not only due to reduction in pump-failure but also to a reduction thrombotic vascular events [The CONSENSUS Trial Study Group 1987; SOLVD 1991]. This reduction in thrombotic vascular events occurs in all cardiovascular conditions treated by ACE inhibition. The HOPE study [2000] showed that treatment of patients with a history of vascular disease, with ramipril, reduced the incidence of cardiovascular death from 17.8% in the placebo group to 14% in the group treated with ramipril. The more recent EUROPA [2003] study showed that treatment with perindopril in patients with 'high risk' vascular disease reduced the incidence of myocardial infarction from 6.2% in patients treated with placebo to 4.8% (RR reduction 22%, CI 10-33%, $p=0.001$)

[EUROPA 2003]. In addition, the recent OPTIMAAL study [2002] compared treatment with the angiotensin receptor blocker losartan and the ACE inhibitor captopril in patients after myocardial infarction. Treatment with captopril reduced the incidence of recurrent myocardial infarction from 15.3% to 13.3% (RR reduction 13%, CI 1-34%, $p=0.03$) when compared to losartan therapy. This evidence confirms that ACE inhibition has benefits above and beyond reduction of angiotensin II that is most likely to be due to the effects of ACE inhibition on bradykinin.

In this thesis it has been shown that bradykinin is an important mediator of vascular protection and repair, due to its effects on endogenous fibrinolysis and vasomotor tone. It has also been shown that these effects can be augmented by ACE inhibition. Further work is required, as outlined above, to elucidate the role of other mediators in preventing ischaemia for patients with atherosclerotic artery disease.

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APPENDIX

PUBLICATIONS ARISING FROM THIS THESIS

Witherow FN, Helmy A, Webb DJ, Fox KA, Newby DE. Bradykinin contributes to the vasodilator effects of chronic angiotensin-converting enzyme inhibition in patients with heart failure. *Circulation* 2001;**104**:2177-81.

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